



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/12, 15/62, A61K 39/35		A2	(11) International Publication Number: WO 93/19178
			(43) International Publication Date: 30 September 1993 (30.09.93)
(21) International Application Number: PCT/US93/02462		(74) Agents: MANDRAGOURAS, Amy, E.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US) et al.	
(22) International Filing Date: 25 March 1993 (25.03.93)			
(30) Priority data:		(81) Designated States: AU, CA, FI, HU, JP, KP, KR, NO, NZ, PT, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
07/857,311 25 March 1992 (25.03.92) US			
07/884,718 15 May 1992 (15.05.92) US			
08/006,116 15 January 1993 (15.01.93) US			
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(54) Title: PEPTIDES USEFUL FOR INDUCING TOLERANCE

Sequence

peptide	name
X	KRDVDFLTGTPDEYVEQVAQYKALPV
Y	KALFVVLENARILKNCVDAMTEEDKE
Z	FFAVANGNELLDLSLTKNATEPER
A	EEDKENALSLLDKIYTSPL
B	MGEAVQNTVEDLKLNTLGR
C	EEDKENALSLLDKIYT
D	NALSLLDKIYTSPL
E	EEDKENALSLLDKIYTSPL

(57) Abstract

The present invention is based on the discovery that subcutaneous administration of a peptide comprising at least one T cell epitope of a protein allergen or other protein antigen results in T cell tolerance upon subsequent exposure to the allergen or other antigen. Peptides useful for inducing tolerance in a mammal, such as a human by subcutaneous administration or other routes of administration are within the scope of this invention. Such peptides include peptide X (SEQ ID NO: 7), peptide Y (SEQ ID NO: 8), peptide Z (SEQ ID NO: 9), peptide A (SEQ ID NO: 10), peptide B (SEQ ID NO: 11), peptide C (SEQ ID NO: 12), peptide D (SEQ ID NO: 13), and peptide E (SEQ ID NO: 14), each as shown in the figure. It is preferred that such peptides be administered in soluble form.

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PEPTIDES USEFUL FOR INDUCING TOLERANCE

Background of the Invention

T lymphocytes can mediate and regulate both the specific and non-specific effector mechanisms of immune responses. CD4+ T lymphocytes provide help
05 for antibody production and secrete cytokines which modulate the growth of other T cells and the growth and differentiation of other immune cells such as monocytes and granulocytes. Functional and biochemical studies have demonstrated that the
10 generation of cellular immune responses depends upon antigen receptors on T cells that recognize peptide fragments of foreign proteins associated with products of the major histocompatibility complex (MHC) that are expressed on antigen-presenting
15 accessory cells.

Experiments with human and mouse T cell clones have demonstrated that the interaction of the T cell receptor and the peptide/MHC protein complex leads to either activation or inactivation of the cell's
20 ability to respond to subsequent peptide/MHC challenges (Lamb, J.R., et al. (1983) J. of Exp. Med. 157:1434-1447; Zander, E.D., et al. (1983) Nature 303:625-627; and Quill, H. and Swartz, R.H. (1987) J. of Immunol. 138:3704-3712). Whether T cell receptor
25 engagement activates or inhibits the T cell's ability to subsequently respond to the peptide/MHC protein complex may be determined by the presence or absence of costimulatory signals delivered to the T cell by the antigen-presenting cell (Jenkins, M.K., et al.
30 (1990) J. of Immunol. 144:1585-1590; and Jenkins, M.K. and Schwartz, R.H. (1987) J. of Exper. Med. 165:302-319). An example is the signal generated by

CD28 binding of B7 (Jenkins, M.K., et al. (1991) The J. of Immunol. 147:2461-2466; Linsley, P.S., et al. (1990) PNAS USA 5031-5035). Costimulatory events of this type appear to be an obligate requirement for T cell activation even when the T cell receptor is bound to an appropriate MHC protein and peptide. Interestingly, if there is no second signal, then the antigen-specific T cells will become tolerized, or enter into a state of anergy. Additionally, in vivo tolerance to antigen challenge has been shown using soluble antigens (Dietrich, F.M. and Weigle, W.O. (1964) J. of Immunol. 92:167-172; Mitchinson, N.A. (1964) Proc. Roy. Soc. B. 161:275-292) or immunogenic peptides administered in such a way as to preclude the second signal (Gammon, G. and Sercarz, E.E. (1989) Nature 342:183-185; Ria, F., et al. (1990) Nature 343:371-383). Peripheral T cell tolerance to endogenous antigens has also been demonstrated in MHC gene transgenic mice in vivo (Burkley, L.C., et al. (1989) Nature 342:564-566, Reviewed in Miller, J.F.A., et al. (1991) Immunol. Reviews 122:103-116 and Hammerling, G.J., et al. (1991) Immunol. Reviews 122:47-67).

Recent advances in technology have made it possible to efficiently culture antigen-specific human and mouse T cell lines and clones in vitro. In addition, it is now possible to produce large amounts of protein antigens or their fragments using recombinant DNA technology or solid phase peptide synthesis. Thus, in the last few years, several research groups have begun to determine the linear amino acid sequences of antigenic proteins that are recognized by T cells in association with MHC (T cell epitopes). Peptides derived from a variety of

protein antigens, including bacterial and viral pathogens, autoantigens, allergens and other experimental antigens such as hen egg lysozyme (HEL), ovalbumin (OVA) and lambda repressor (cl) have been examined for the ability to stimulate antigen-specific T cells. A wide spectrum of peptides has been reported to serve as T cell epitopes. For example, OVA amino acid residues 324-339 (Shimonkevitz, R. et al., J. Immunol., 133:2167 (1984)), HEL amino acid residues 74-96 (Shastri, N. et al., J. Exp. Med. 164:882-896 (1986); and lambda repressor (cl) amino acid residues 12-26 (Lai, M.-Z et al., J. Immunol., 139:3973-3980 (1987)) have been demonstrated to stimulate whole protein-primed T cells.

In addition, a peptide derived from Hepatitis B surface antigen (HBsAg amino acid residues 19-33) has recently been shown to stimulate T cell responses in a majority of human subjects who had been immunized with a recombinant hepatitis B vaccine (Schad, V.C. et al., Seminars in Immunol., 3:217-224 (1991)). A major mycobacterial antigen 65-kD protein has also been epitope-mapped (Lamb, J.R. et al., EMBO J., 6(5):1245-1249 (1987)). T cell epitopes have been identified in the peptides comprised of amino acid residues 112-132 and 437-459 of the 65-kD protein. Myelin basic protein (MBP), an autoantigen which induces experimental autoimmune encephalomyelitis (EAE) and the presumed autoantigen in multiple sclerosis (MS) has also been epitope-mapped in both human (Ota, K. et al., Nature, 346:183-187 (1990)) and rodent (Zamvil et al., Nature 324:258-260(1986)) systems. Ota et al. have identified a major T cell epitope recognized by MS patients, MBP amino acid

residues 84-102. Minor epitopes (MBP amino acid residues 143-168, 61-82, 124-42 and 31-50) recognized by T cells from MS patients were also described. Zamvil et al. have shown that MBP amino acid residues 1-11 contain the major T cell epitope(s) causing EAE, in susceptible rodent strains.

T cell epitopes present in allergenic proteins have very recently been described (O'Hehir, R. et al., Ann. Rev. Immunol., 9:67-95 (1991)). Several peptides derived from the house dust mite allergen Der p I have been shown to be T cell-reactive (Thomas, W.R., et al., In Epitopes of Atopic Allergens Proceedings of Workshop from XIV Congress of the European Academy of Allergy and Clinical Immunology, Berlin (Sept. 1989) pp. 77-82; O'Hehir, R.E. Annual Review Immunology 9:67-95 (1991); Stewart, G.A. et al., In: Epitopes of Atopic Allergens Proceedings of Workshop from XIV Congress of the European Academy of Allergy and Clinical Immunology, Berlin (Sept. 1989) pp 41-47; and Yessel, H. et al., In: T Cell Activation in Health and Disease: Discrimination Between Immunity and Tolerance, Conference 22-26 (Sept. 1990) Trinity College, Oxford U.K.). A T cell-stimulatory peptide derived from the short ragweed allergen Amb a I amino acid residues 54-65 has also been reported (Rothbard, J.B. et al., Cell, 52: 515-523 (1988)). Using a panel of T cell clones derived from a rye grass-allergic individual, Perez et al. demonstrated that T cell epitopes are contained within amino acid residues 191-210 of the protein allergen Lol p I (Perez, M. et al., J. Biol. Chem., 265(27):16210-16215 (1990)).

Summary of the Invention

Effective intervention in allergic and other immune response mediated diseases requires altering an established or ongoing immune response. The present invention is based on the discovery that subcutaneous administration of a peptide to a mammal decreases the T cell response to subsequent challenge with the same peptide, whether a mammal is naive or preexposed to the protein allergen or other antigen from which the peptide is derived. This invention also relates to peptides useful for tolerization by subcutaneous or other routes of administration. For such tolerization to be clinically useful, it must be effective upon environmental exposure to whole protein allergen or other protein antigen. Thus, subcutaneous administration of a peptide comprising at least one T cell epitope of a protein allergen or other protein antigen results in T cell tolerization or anergy upon exposure to the allergen or other antigen. Such peptide-induced T cell tolerance can be used to treat immune mediated diseases such as allergy. The ability to tolerize with subcutaneous injection of a peptide rather than intravenous or intraperitoneal injection provides a more practical and efficient therapeutic approach.

Peptides useful in methods of tolerizing a mammal, such as a human can be derived from protein allergens. These peptides comprise at least one T cell epitope of an allergen and preferably have minimal immunoglobulin E stimulating activity. In addition, for therapeutic purposes, it is preferred that such peptides bind immunoglobulin E to a substantially lesser extent than the protein allergen from which the peptides is derived binds immunoglobulin E. More preferably, peptides derived

from protein allergens do not bind immunoglobulin E specific for the protein allergen in a substantial percentage (at least about 75%) of a population of individuals sensitive to the protein allergen, or if
05 such binding occurs, such binding does not result in mediator release e.g., histamine, from mast cells or basophils.

The present invention also relates to subcutaneous administration of a peptide derived from
10 an autoantigen, such as insulin, myelin basic protein and acetylcholine receptors. These peptides preferably do not bind immunoglobulin specific for the autoantigen in a substantial percentage (at least about 75%) of a population of individuals sensitive
15 to the autoantigen. Further, peptides derived from protein allergens or other protein antigens can be designed such that an undesirable property of the native protein (e.g., enzymatic activity) can be eliminated for therapeutic purposes. In addition,
20 for increased therapeutic efficacy, peptides as described herein are preferably administered in soluble form, subcutaneously, to a mammal to tolerize T cells of the mammal to the protein antigen from which the peptide is derived.

25 Peptides for treating sensitivity to Felis domesticus which are derived from the T Cell Reactive Feline Protein (TRFP) and which are useful for tolerization are within the scope of the invention. Compositions comprising one or more of the following
30 peptides can be administered to an individual sensitive to Felis domesticus: peptide X (SEQ ID NO: 7); peptide Y (SEQ ID NO: 8); peptide Z (SEQ ID NO: 9); peptide A (SEQ ID NO: 10); peptide B (SEQ ID NO: 11); peptide C (SEQ ID NO: 12); peptide D (SEQ ID NO:

13); and peptide E (SEQ ID NO: 14) of TRFP. Methods of treating sensitivity to Felis domesticus in an individual comprising administering to the individual a therapeutically effective amount of one or more
05 therapeutic compositions comprising at least one of such peptides are also within the scope of the invention.

Brief Description of the Drawings

Figure 1 is the nucleic acid sequence and
10 deduced amino acid sequence of chain 1 of the human T Cell Reactive Feline Protein (TRFP) including leader sequences A (SEQ ID NO: 1 and 2) and B (SEQ ID NO: 3 and 4).

Figure 2 is the nucleic acid sequence and
15 deduced amino acid sequence of chain 2 of TRFP including a leader sequence (SEQ ID NO: 5 and 6).

Figure 3 is the amino acid sequences of peptide X (SEQ ID NO: 7), peptide Y (SEQ ID NO: 8), peptide Z (SEQ ID NO: 9), peptide A (SEQ ID NO: 10) and peptide
20 B (SEQ ID NO: 11), peptide C (SEQ ID NO: 12), peptide D (SEQ ID NO: 13) and peptide E (SEQ ID NO: 14) of TRFP, each of which contains at least one T cell epitope of TRFP.

Figure 4 is a graphic representation of the
25 induction of T cell tolerance in mice by administration of peptide Y subcutaneously or intravenously followed by challenge with peptide Y. Lymph node cells were isolated, cultured in vitro with peptide Y and tested for the presence of IL-2.

30 Figure 5 is a graphic representation of the dose response necessary to induce T cell tolerance with subcutaneous administration of peptide Y in mice.

Figure 6 is a graphic representation of the dose response necessary to induce T cell tolerance with subcutaneous administration of peptide X in mice.

05 Figure 7 is a graphic representation of the induction of T cell tolerance in mice by administration of a combination of peptide X and peptide Y subcutaneously followed by challenge with both peptide X and peptide Y. Lymph node cells were isolated, cultured in vitro with a combination of
10 peptide X and peptide Y or with peptide X or peptide Y separately and tested for the presence of IL-2.

Figure 8 is a graphic representation of the response of mice preimmunized with TRFP upon administration of peptide Y.

15 Figure 9A is a graphic representation of the induction of T cell tolerance in mice primed with TRFP and tolerized with peptide Y. Lymph node cells were isolated, cultured in vitro with peptide Y and tested for the presence of IL-2. Figure 9B is a
20 graphic representation of the induction of IgG tolerance in mice primed with TRFP and tolerized with peptide Y. A sera sample was obtained and assayed for binding to peptide Y in a standard ELISA assay.

Figures 10A and 10B are graphic representations
25 of the induction of T cell tolerance in mice primed with TRFP and tolerized with peptide Y. Lymph node cells were isolated, cultured in vitro with peptide Y and tested for the presence of IL-2 (culture with CTLL-3) or IL-4 (culture with CT4S).

30 Figure 11 is a graphic representation of the induction of T cell tolerance in mice with recombinant chain 1 of TRFP (peptide Y and peptide X) peptides Fel 3-1, peptide Y, peptide C and peptide D. The mice were challenged with recombinant chain 1

of TRFP. Lymph node cells were isolated, cultured with recombinant chain 1 of TRFP and tested for the presence of IL-2.

5 Figure 12 is a graphic representation of the results of direct binding studies of human IgE to TRFP, peptide X and peptide Y.

 Figure 13 is a graphic representation of the results of histamine release from basophils in response to TRFP, peptide X and peptide Y.

10 Figure 14 shows the amino acid sequences of the following peptides: Fel 33, Fel 34, Fel 35, Fel 36, Fel 37, Fel 38, Fel 38-1, Fel 39 and Fel 39.1.

 Figure 15A, B and C is a graphic representation depicting the response of T cells from patient #688 primed in vitro to TRFP and analyzed for response to various peptides derived from TRFP as measured by IL-2 production and IL-4 production.

 Figure 16A, B and C is a graphic representation depicting the response of T cells from patient #730 primed in vitro to TRFP and analyzed for response to various peptides derived from TRFP as measured by IL-2 production and IL-4 production.

 Figure 17A, B and C is a graphic representation depicting the response of T cells from patient #738 primed in vitro to TRFP and analyzed for response to various peptides derived from TRFP as measured by IL-2 production and IL-4 production.

 Figure 18A, B and C is a graphic representation depicting the response of T cells from patient #807 primed in vitro to TRFP and analyzed for response to various peptides derived from TRFP as measured by IL-2 production and IL-4 production.

 Figure 19A, B and C is a graphic representation depicting the response of T cells from patients #688, #730,

#738, and #807 primed in vitro to TRFP and analyzed for response to various peptides derived from TRFP as measured by Il-2 production and Il-4 production.

05 Figure 20 is a graph depicting the ratio of Il-4 production to Il-2 production by T cell lines from cat allergic patients, #688, #730, #738, and #807 in response to various peptides derived from TRFP.

10 Figure 21 is a graph depicting the results of a direct ELISA assay in which various antigens were incubated with human plasma obtained from a cat allergic individual.

15 Figure 22 is a graph depicting the results of a depletion ELISA assay in which human plasma obtained from a cat allergic individual was preabsorbed on various antigens followed by incubation on fresh antigen coated plates.

Detailed Description of the Invention

The present invention is based on the discovery that subcutaneous administration of a peptide
20 comprising at least one T cell epitope of a protein allergen or other protein antigen results in T cell tolerance upon subsequent exposure to the allergen or other antigen. Peptides useful for inducing tolerance in a mammal, such as a human by
25 subcutaneous administration or other routes of administration are within the scope of this invention. Such peptides include peptide X (SEQ ID NO: 7), peptide Y (SEQ ID NO: 8), peptide Z (SEQ ID NO: 9), peptide A (SEQ ID NO: 10), peptide B (SEQ ID
30 NO: 11), peptide C (SEQ ID NO: 12), peptide D (SEQ ID NO: 13), and peptide E (SEQ ID NO: 14), each as shown in Figure 3. It is preferred that such peptides be administered in soluble form.

In general, peptides useful in methods of tolerization have human T cell stimulating activity as determined by standard T cell biology techniques and, thus, comprise at least one T cell epitope. If
05 desired, precise T cell epitopes can be determined by, for example, fine mapping techniques. This technique involves modifying a peptide comprising at least one T cell epitope as defined by standard T
10 cell biology techniques by addition or deletion of amino acid residues at either the amino or carboxy terminus of the peptide. Following modification, the peptide is tested to determine a change in T cell reactivity. If two or more peptides derived from a
15 protein antigen share an area of overlap and are both found to have human T cell stimulating activity (as determined by standard T cell biology techniques) additional peptides can be produced comprising all or a portion of such peptides and these additional
20 peptides can be tested by the above fine mapping procedure. As a result of fine mapping, a set of human T cell epitopes for a protein allergen or other antigen comprising amino acid residues essential to T cell recognition can be produced.

In the situation where the T cell epitopes of a
25 protein allergen or other protein antigen are unknown or ill-defined (e.g., some or all of the peptide regions of the protein antigen which have human T cell stimulating activity have not been defined by standard T cell biology techniques or the precise
30 human T cell epitopes of the protein antigen have not been defined by fine mapping techniques) a peptide useful in methods of subcutaneous tolerization may be obtained by reviewing the known protein structure of an allergen or other antigen and theoretically

dividing the allergen or antigen into at least two peptide regions of desired lengths. For example, the protein sequence of the allergen or other antigen can be systematically divided into at least two
05 non-overlapping peptide regions of desired lengths, or at least two overlapping peptide regions of desired lengths. This division into peptide regions can be arbitrary, can be made according to an algorithm, or can be wholly or partially based on
10 regions of the protein antigen known to comprise at least one T cell epitope.

When just a few of the peptide regions of the protein allergen or other protein antigen comprising at least one T cell epitope are known or when all the
15 regions of the protein allergen or other protein antigen which have human T cell stimulating activity are unknown, preferably, at least 50% of the entire protein sequence of the protein allergen or other protein antigen and more preferably, the entire
20 protein sequence of the protein allergen or other protein antigen is divided into one or more peptides. The peptide or peptides can then be produced recombinantly or synthetically and the ability of the peptide(s) to stimulate human T cells
25 can be determined. A peptide derived from a protein allergen can be tested to determine whether the peptide binds immunoglobulin E specific for the allergen and result in the release of mediators (e.g., histamine) from mast cells or basophils.
30 Those peptide(s) found to bind immunoglobulin E and cause the release of mediators from mast cells or basophils in greater than approximately 10-15% of the allergic sera tested are preferably not used in methods of the invention.

and cross-linking of antigen to IgE on mast cells or basophils and the release of mediators (e.g., histamine, serotonin, eosinophil chemotactic factors). Thus, anaphylaxis could be avoided by the use of a peptide which does not bind IgE, or if the peptide binds IgE, such binding does not result in the release of mediators (e.g., histamine, etc.) from mast cells or basophils. In addition, peptides which have minimal IgE stimulating activity are particularly desirable for therapeutic effectiveness. Minimal IgE stimulating activity refers to IgE production that is less than the amount of IgE production and/or IL-4 production stimulated by the whole protein allergen.

Peptides useful in methods of the invention as well as the preferred peptides derived from TRFP (e.g., peptide X, peptide Y, etc.) can be produced by recombinant DNA techniques in a host cell transformed with a nucleic acid vector directing expression of a nucleotide sequence coding for such peptide, or by chemical synthesis, or in certain limited situations by chemical cleavage of a protein allergen or other protein antigen. When produced by recombinant techniques, host cells transformed with nucleic acid vectors directing expression of a nucleotide sequence coding for a peptide are cultured in a medium suitable for the cells. The peptides may be secreted and harvested from a mixture of cells and cell culture medium. Alternatively, the peptide may be retained cytoplasmically and the cells harvested, lysed and the peptide isolated and purified. Peptides can be isolated using techniques known in the art for purifying peptides or proteins including ion-exchange chromatography, gel filtration

chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for the peptide, the protein allergen or other antigen from which the peptide is derived, or a
05 portion thereof. Any of the peptides described herein are isolated such that the peptide is substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or substantially free of chemical precursors or other
10 chemicals when synthesized chemically, or obtained by chemical cleavage of a protein allergen or other protein antigen.

According to one aspect of the invention, peptides derived from a protein allergen or other
15 antigen, are administered, preferably subcutaneously to a mammal (such as a human) sensitive to the protein allergen or antigen in a form which results in a decrease in the T cell response of the mammal upon subsequent exposure to the allergen or other
20 antigen. As used herein, a decrease or modification of the T cell response of a mammal sensitive to a protein allergen or other antigen can be defined as non-responsiveness or diminution in symptoms to the allergen or other antigen in the mammal, as
25 determined by standard clinical procedures (see e.g., Varney et al., British Medical Journal 302: 265-269 (1990)), including diminution in allergen induced asthmatic symptoms. As referred to herein, a diminution in symptoms to an allergen includes any
30 reduction in the allergic response of a mammal, such as a human, to the allergen following a treatment regimen with a peptide as described herein. This diminution in symptoms may be determined subjectively

in a human (e.g., the patient feels more comfortable upon exposure to the allergen), or clinically, such as with a standard skin test.

As a result of the work described herein,
05 peptides derived from protein allergens or other protein antigens and having T cell stimulating activity (i.e., having at least one T cell epitope) have been produced, for example, the preferred peptides for use in treating sensitivity to Felis
10 domesticus and derived from TRFP (i.e., peptide X, peptide Y, peptide Z, peptide A, peptide B, peptide C, peptide D, peptide E). T cell epitopes are believed to be involved in initiation and perpetuation of the immune response to a protein
15 allergen or other protein antigen which is responsible respectively for the clinical symptoms of allergy or other diseases. These T cell epitopes are thought to trigger early events at the level of the T helper cell by binding to an appropriate HLA molecule
20 on the surface of an antigen presenting cell and stimulating the relevant T cell subpopulation. These events lead to T cell proliferation, lymphokine secretion, local inflammatory reactions, recruitment of additional immune cells to the site, and
25 activation of the B cell cascade leading to production of antibodies. One isotype of these antibodies, IgE, is fundamentally important to the development of allergic symptoms and its production is influenced early in the cascade of events, at the
30 level of the T helper cell, by the nature of the lymphokines secreted. A T cell epitope is the basic element or smallest unit of recognition by a T cell receptor, where the epitope comprises amino acids essential to receptor recognition and may be

contiguous and/or non-contiguous in the amino acid sequence of the protein. A T cell epitope, as used herein has a stimulation index of at least 2.0, more preferably at least 2.5, even more preferably at least 3.5 and most preferably at least 5.0.

According to the present method, subcutaneous administration of a peptide as described herein to a mammal, such as a human, will tolerize or anergize appropriate T cell subpopulations such that they become unresponsive to the protein allergen or other antigen and do not participate in stimulating an immune response upon such exposure (see Exemplification A). In addition, administration of such a peptide may modify the lymphokine secretion profile as compared with exposure to the naturally-occurring protein allergen or portion thereof (e.g., result in a decrease of IL-4 and/or an increase in IL-2). Furthermore, exposure to the peptide may influence T cell subpopulations which normally participate in the response to the allergen such that these T cells are drawn away from the site(s) of normal exposure to the allergen (e.g., nasal mucosa, skin, and lung) towards the site(s) of therapeutic administration of the peptide. This redistribution of T cell subpopulations may ameliorate or reduce the ability of an individual's immune system to stimulate the usual immune response at the site of normal exposure to the allergen, resulting in a diminution in allergic symptoms.

Peptides useful for tolerization can comprise as many amino acid residues as desired and preferably comprise at least about 7, more preferably at least about 15, even more preferably at least about 20 and most preferably at least about 25 amino acid residues

of a protein allergen or other protein antigen. A peptide length of about 20-40 amino acid residues is preferred as increases in length of a peptide may result in difficulty in peptide synthesis as well as retention of an undesirable property (e.g., immunoglobulin binding or enzymatic activity) due to maintenance of conformational similarity between the peptide and the protein allergen or other protein antigen from which it is derived. If desired, the amino acid sequences of one or more peptides can be produced and joined by a linker to increase sensitivity to processing by antigen-presenting cells. Such linker can be any non-epitope amino acid sequence or other appropriate linking or joining agent.

When peptides as used to tolerize an individual sensitive to a protein allergen by subcutaneous administration, the peptide is preferably derived from a protein allergen of the following genus: the genus Dermatophagoides; the genus Felis; the genus Ambrosia; the genus Lolium; the genus Cryptomeria; the genus Alternaria; the genus Alder; the genus Betula; the genus Quercus; the genus Olea; the genus Artemisia; the genus Plantago; the genus Parietaria; the genus Canis; the genus Blattella, the genus Apis; and the genus Periplaneta. For example, peptides useful for subcutaneous tolerization can be derived from known or unknown protein allergens including those of the following species: Dermatophagoides pteronyssinus (e.g., Der p I, Der p II); Dermatophagoides farinae (e.g., Der f I, Der f II); Ambrosia artemisiifolia (e.g., Amb a I.1, Amb a I.2, Amb a I.3, Amb a I.4, Amb a II); Cryptomeria japonis (e.g., Cry j I, Cry j II); Lolium perenne (e.g. Lol p

I, Lol p IX); Felis domesticus (e.g., Fel d I); and Canis familiaris (e.g., Can f I, Can f II). Peptides comprising at least one epitope of protein allergens have been described in the following applications, the contents of which are incorporated herein by reference: U.S.S.N. 866,679 entitled "T Cell Epitopes of the Major Allergens from Ambrosia Artemisiifolia" filed April 9, 1992; and U.S.S.N. 963,381 entitled "T Cell Epitopes of the Major Allergens of Dermatophagoides (House Dust Mite) filed October 16, 1992.

Particularly preferred peptides for treating sensitivity to Felis domesticus are derived from the genus Felis and include the following: peptide X (SEQ ID NO: 7); peptide Y (SEQ ID NO: 8); peptide Z (SEQ ID NO: 9); peptide A (SEQ ID NO: 10); peptide B (SEQ ID NO: 11), peptide C (SEQ ID NO: 12); peptide D (SEQ ID NO: 13); and peptide E (SEQ ID NO: 14), of TRFP, the amino acid sequences of each peptide as shown in Fig. 3, and modifications thereof. These preferred peptides from TRFP can be produced recombinantly or by chemical synthesis as herein described based on the nucleic acid and amino acid sequences of TRFP shown in Figs. 1 and 2 (SEQ ID NO: 1-6). Such peptides can be administered in the form of a therapeutic composition to treat sensitivity to Felis domesticus. Thus, one or more of peptides X, Y, Z, A, B, C, D and E can be combined in a single composition or one or more separate compositions for simultaneous or sequential administration to an individual to be treated for sensitivity to Felis domesticus. Such compositions can be administered subcutaneously or otherwise, including, but not limited to intravenously and intraperitoneally in a

form suitable for tolerization. Preferably, the peptide is administered in soluble form.

Additional peptides useful for subcutaneous tolerization can be derived from protein antigens
05 other than protein allergens where modification of an antigen specific immune response is desired. For example, a known autoantigen involved in the pathogenesis of an autoimmune disease can be examined and peptides having human T cell stimulating activity
10 or peptides having known T cell epitopes can be identified. One or more of such peptides can be administered subcutaneously to a mammal, such as a human, afflicted with an autoimmune disease or at risk of developing an autoimmune disease to decrease
15 the antibody response to the autoantigen, to interfere with efficacy and/or decrease immune complex related side effects. In order to preserve the T cell reactivity of the autoantigen, peptides of the autoantigen having human T cell stimulating
20 activity can be defined by standard T cell biology techniques, or if desired, precise T cell epitopes can be defined by above-described fine mapping techniques.

Peptides which stimulate T cells and do not have
25 undesired properties associated with the autoantigen (e.g., do not bind autoantibodies in a substantial percentage of individuals sensitive to the autoantigen) are selected for use as immunotherapeutics to tolerize an individual to the
30 autoantigen. In the form of a therapeutic composition, the peptide would be delivered subcutaneously in a physiologically acceptable vehicle in the absence of adjuvant to allow the peptide to induce antigen specific tolerance to the

autoantigen from which the peptide is derived and regulate any potentially damaging immune response. Among autoantigens useful in producing peptides are insulin, glutamic acid decarboxylase (64K), PM-1 (a
05 69KD autoantigen) and carboxypeptidase in diabetes; myelin basic protein in multiple sclerosis; rh factor in erythroblastosis fetalis; acetylcholine receptors in myasthenia gravis; thyroid receptors in Graves' Disease; basement membrane proteins in Good Pasture's
10 syndrome; and thyroid proteins in thyroiditis. For example, known regions of myelin basic protein (MBP) having human T cell stimulating activity include a region comprising all or a portion of amino acid residues 84-106 (preferably amino acid residues
15 84-102 and more preferably amino acid residues 89-101) of human MBP and a region comprising all or a portion of amino acid residues 140-172 (preferably amino acid residues 143-168 of human MBP). One or more peptides comprising each of these regions could
20 be produced and administered subcutaneously to treat multiple sclerosis in an individual.

Peptides useful in methods of treating sensitivity in a mammal, such as a human, to a protein allergen (including the preferred peptides X,
25 Y, Z, etc. derived from TRFP) or other protein antigen are in the form of a therapeutic composition. Such compositions include a peptide and a pharmaceutically acceptable carrier or diluent. Administration of the therapeutic compositions of the
30 present invention to desensitize or tolerize an individual to a protein allergen or other protein antigen can be carried out using procedures, at dosages and for periods of time effective to reduce sensitivity (i.e., reduce the allergic response) of

the individual to a protein allergen or other protein antigen. Effective amounts of the therapeutic compositions will vary according to factors such as the degree of sensitivity of the individual to the allergen or other antigen, the age, sex, and weight of the individual, and the ability of the peptide(s) to elicit an antigenic response in the individual. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

Peptides derived from TRFP used in treating sensitivity to Felis domesticus (e.g., peptide X, peptide Y, etc.) may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound may be coated with in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

To administer a peptide or peptides of the invention by other than parenteral administration, it may be necessary to coat the peptide with, or co-administer the protein or peptide with, a material to prevent its inactivation. For example, the peptides may be administered to an individual in an appropriate diluent or adjuvant, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Adjuvant is used in its broadest sense and includes

any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme
05 inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan et al., (1984) J. Neuroimmunol. 7:27). For purposes of
10 inducing T-cell anergy, the therapeutic composition is preferably administered in non-immunogenic form, e.g., one that does not contain adjuvant.

The active compound may also be administered parenterally or intraperitoneally. Dispersions can
15 also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

20 Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases,
25 the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as
30 bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable

oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

- 05 Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, asorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic
10 agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for
15 example, aluminum monostearate and gelatin.

- Sterile injectable solutions can be prepared by incorporating active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required,
20 followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case
25 of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (i.e., a peptide of the invention) plus any additional desired
30 ingredient from a previously sterile-filtered solution thereof.

When a preferred peptide or peptides derived from TRFP, as herein described is suitably protected, as described above, the peptide may be orally

administered, for example, with an inert diluent or an assimilable edible carrier. The peptide and other ingredients may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or
05 incorporated directly into the individual's diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups,
10 wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the
15 weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit
20 contains between from about 10 μ g to about 200 mg of active compound.

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents,
25 isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use
30 thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease

of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit
05 containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by
10 and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieve, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of
15 sensitivity in individuals.

Therapeutic compositions of the invention can be administered subcutaneously to mammals, such as humans, sensitive to an allergen or other protein antigen from which the peptide is derived, at dosages
20 and for lengths of time effective to reduce sensitivity in the mammal to the allergen or other antigen. For example, an amount of one or more of the same or of different therapeutic compositions effective to tolerize T cells of an individual is
25 administered subcutaneously, simultaneously or sequentially. A composition comprising at least two peptides (e.g., a physical mixture of at least two peptides), can also be used in methods of subcutaneous tolerization.

30 It is also possible to modify the structure of peptides useful in methods of the invention for such purposes as increasing solubility, enhancing therapeutic or preventive efficacy, or stability (e.g., shelf life ex vivo, and resistance to

proteolytic degradation in vivo). A modified peptide can be produced in which the amino acid sequence has been altered, such as by amino acid substitution, deletion, or addition, to modify immunogenicity
05 and/or reduce allergenicity, or to which a component has been added for the same purpose. For example, the amino acid residues essential to T cell epitope function can be determined using known techniques (e.g., substitution of each residue and determination
10 of presence or absence of T cell reactivity). Those residues shown to be essential can be modified (e.g., replaced by another amino acid whose presence is shown to enhance T cell reactivity), as can those which are not required for T cell reactivity (e.g.,
15 by being replaced by another amino acid whose incorporation enhances T cell reactivity but does not diminish binding to relevant MHC). Another example of a modification of peptides is substitution of cysteine residues preferably with alanine, or
20 glutamic acid, or alternatively with serine or threonine to minimize dimerization via disulfide linkages.

In order to enhance stability and/or reactivity, peptides can also be modified to incorporate one or
25 more polymorphisms in the amino acid sequence of a protein allergen resulting from natural allelic variation. Additionally, D-amino acids, non-natural amino acids or non-amino acid analogues can be substituted or added to produce a modified peptide
30 within the scope of this invention. Furthermore, peptides can be modified using the polyethylene glycol (PEG) method of A. Sehon and co-workers (Wie et al. supra) to produce a peptide conjugated with PEG. Modifications of peptides can also include

reduction/alkylation (Tarr in: Methods of Protein Microcharacterization, J.E. Silver ed. Humana Press, Clifton, NJ, pp 155-194 (1986)); acylation (Tarr, supra); esterification (Tarr, supra); chemical
05 coupling to an appropriate carrier (Mishell and Shiigi, eds, Selected Methods in Cellular Immunology, WH Freeman, San Francisco, CA (1980); U.S. Patent 4,939,239); or mild formalin treatment (Marsh
10 International Archives of Allergy and Applied Immunology 41: 199-215 (1971)).

To facilitate purification and potentially increase solubility of peptides, it is possible to add reporter group(s) to the peptide backbone. For example, poly-histidine can be added to a peptide to
15 purify the peptide on immobilized metal ion affinity chromatography (Hochuli, E. et al., Bio/Technology, 6:1321-1235 (1988)). In addition, specific endoprotease cleavage sites can be introduced, if desired, between a reporter group and amino acid
20 sequences of a peptide to facilitate isolation of peptides free of irrelevant sequences. In order to successfully desensitize an individual to a protein antigen, it may be necessary to increase the solubility of a peptide by adding functional groups
25 to the peptide or by not including hydrophobic T cell epitopes or regions containing hydrophobic epitopes in the peptide.

To potentially aid proper antigen processing of T cell epitopes within a peptide, canonical protease
30 sensitive sites can be recombinantly or synthetically engineered between regions, each comprising at least one T cell epitope. For example, charged amino acid pairs, such as KK or RR, can be introduced between regions within a peptide during recombinant

construction of the peptide. The resulting peptide can be rendered sensitive to cathepsin and/or other trypsin-like enzymes cleavage to generate portions of the peptide containing one or more T cell epitopes.

05 In addition, such charged amino acid residues can result in an increase in solubility of a peptide.

Site-directed mutagenesis of DNA encoding a peptide can be used to modify the structure of the peptide. Such methods may involve PCR (Ho et al.,
10 Gene 77:51-59 (1989)) or total synthesis of mutated genes (Hostomsky, Z., et al., Biochem. Biophys. Res. Comm. 161:1056-1063 (1989)). To enhance bacterial expression, the aforementioned methods can be used in conjunction with other procedures to change the
15 eucaryotic codons in DNA constructs encoding peptides to ones preferentially used in E. coli.

Another aspect of the invention provides peptides shown to have T cell stimulating activity and thus comprising at least one T cell epitope which
20 can be administered to an individual in the form of a therapeutic composition to reduce the individual's response to an allergen of Felis domesticus. Such a therapeutic composition can comprise one or more of such peptides. A preferred composition comprises at
25 least one peptide selected from the group consisting of peptide X (amino acid residues 7-30 from chain 1 of TRFP; also referred to as Fel 8-3 and shown in Figure 3); peptide Y (amino acid residues 29-55 from chain 1 of TRFP; also referred to as Fel 30-4 and
30 shown in Figure 3); peptide Z (amino acid residues 14-39 from chain 2 of TRFP; also referred to as Fel 31-2 and shown in Figure 3); peptide A (amino acid residues 51-69 from chain 1 of TRFP and shown in Figure 3); peptide B (amino acid residues 74-92 from

chain 2 of TRFP; also referred to as Fel 29 and shown in Figure 3); peptide C (amino acid residues 51-66 from chain 1 of TRFP, also referred to as Fel 23 and shown in Figure 3); peptide D (amino acid residues 56-69 from chain 1 of TRFP; a modified form of Fel 21 and shown in Figure 3); and peptide E (amino acid residues 50-69 from claim 1 of TRFP and shown in Figure 3).

As described in detail in Exemplification A, T cell tolerance has been induced in mice by subcutaneous or intravenous administration of peptide X or peptide Y separately or in combination. The tolerization of the mice was evidenced by decreases in peptide specific IL-2 production, IL-4 production, and antibody production. Decreases in the T cell activities that are expected to be associated with IgE production, T cell help for B cells and IL-4 production have been shown. In addition, T cells specific for recombinant chain 1 of TRFP were tolerized by administering peptide X and peptide Y.

In another set of experiments, T cell tolerance was induced to myelin basic protein (MBP) in mice by (1) administering one or a combination of two immunodominant T cell determinants from MBP (i.e., a.a. residues 35-47 and Ac 1-11) prior to immunization with MBP and (2) administering one or a combination of the immunodominant T cell determinants following induction of Experimental Allergic Encephalomyelitis (EAE) in the mice. Tolerance induction prior to challenge with MBP blocked the development of severe disease following MBP immunization. "Tolerizing" mice once disease had begun, 10 days following immunization with MBP, blocked disease progression and decreased severity of ongoing disease.

The invention further encompasses at least one therapeutic composition useful in treating a disease which involves an immune response to protein antigen (e.g., an allergen, an autoantigen, etc.) comprising
05 at least one peptide having a sufficient percentage of the T-cell epitopes of the protein antigen such that in a substantial percentage of a population of individuals sensitive to the protein antigen, the response of such individuals to the protein antigen
10 is substantially diminished, with the proviso that the at least one peptide does not comprise the entire protein antigen.

This invention is further illustrated by the following non-limiting examples.

15 EXEMPLIFICATION A

Peripheral T Cell Tolerance Induced in Naive & Primed Mice By Subcutaneous Injection of Peptides From the Human T Cell Reactive Feline Protein (TRFP)

In each of the following experiments the protein
20 and peptides were produced as follows. An overlapping set of peptides derived from the sequence of Fel d I chain 1 (referred to herein as Human T Cell Reactive Feline Protein (TRFP) chain 1) (Morgenstern, J.P. et al., Proc. Natl. Acad. Sci. (1991) 88:9690-9694)) was synthesized using
25 conventional Merrifield solid phase t-Boc or Fmoc chemistry. The peptides were purified by reverse phase HPLC and examined by amino acid analysis. The lyophilized peptides were resuspended in PBS and
30 sterilized by gamma irradiation (10,000 rads) or by passage through a 0.2 μ filter.

Native TRFP protein was purified from an extract of house dust as described by Chapman et al. (J. Immunol. (1988) 140:812-818)). Briefly, house dust (from vacuum containers used in homes with multiple cats) was extracted with PBS, then lyophilized and redissolved in water. The extract was applied to a column coupled with anti-TRFP monoclonal antibody (hybridomas 6F9 and 1G4 were both provided M. Chapman). The TRFP was eluted from the column with 100 mM glycine pH 2.5 and was neutralized. Recombinant TRFP chain 1 was expressed and purified as described in Rogers et al. (Rogers, B.L. et al., International Symposium - Workshop, Molecular Biology and Immunology of Allergens (1992), CRC-Press, Inc. (in Press)). TRFP chain 1 was approximately 96% pure and contains the amino acids glycine and serine from a thrombin cleavage recognition site and the N-terminus followed by the complete 70 amino acid TRFP chain 1 sequence (Ohman, J.L. et al., J. Immunol. (1974) 113:1668-1677).

Example 1

Subcutaneous peptide inhibits the response to subsequent peptide challenge

To assess the ability of subcutaneously administered peptide to affect a T cell response, two groups of five B6CBAF₁ mice (Age matched B6D2F₁ (H-2^{bxa}), Jackson Lbas, Bar Harbor, ME) 6-10 week old female mice) were treated with subcutaneous injections of either peptide Y in PBS or PBS alone. B6CBAF₁ mice were chosen because of their strong T cell response to peptide Y. The mice were then challenged with peptide Y in CFA. A peptide specific T cell response was measured by peptide Y-specific

IL-2, IL-4, and IFN- γ production 10 days after challenge. As shown in Table 1, peptide Y specific production of all of the examined cytokines by lymph node or spleen cells was decreased in cells from the
05 peptide-tolerized animals. Note that in each of the cytokine assays there were similar low backgrounds using cells from both groups of animals in cultures with no peptide Y. This experiment demonstrates that subcutaneous peptide injection can induce peptide
10 specific T cell tolerance as shown by the inability of the cells to specifically produce cytokines. Note that production of IL-4 was decreased in the cultures from peptide-tolerized animals. In addition, antigen-specific T cell proliferation was decreased
15 in the peptide-tolerized animals

Table 1. Tolerization by s.c. injection with a peptide decreases the T cell response to that peptide.

Subcutaneous tolerization*	cells†	($\mu\text{g/ml}$) In vitro IPC-2	(cpm $\times 10^{-3}$) IL-2	(cpm $\times 10^{-3}$) IL-4	(O.D. 450nm) IFN- γ
PBS	lymph node	150	49.86 (6.46)‡	2.82 (0.29)	1.20 (0.02)
		0	2.61 (0.98)	0.56 (0.03)	0.06 (0.01)
	spleen	150	31.62 (4.54)	13.31 (2.96)	1.14 (0.01)
		0	2.72 (0.85)	1.17 (0.14)	0.06 (0.01)
IPC-2	lymph node	150	6.82 (0.64)	1.04 (0.08)	0.26 (0.11)
		0	2.60 (0.74)	0.39 (0.03)	0.08 (0.01)
	spleen	150	4.53 (0.32)	1.09 (0.20)	0.09 (0.01)
		0	1.60 (0.70)	1.09 (0.11)	0.07 (0.01)

* Five naive B6CBAF₁ mice were tolerized by subcutaneously administering either IPC-2 peptide in PBS or PBS alone. They were then challenged with IPC-2.

† Draining lymph nodes and spleens were removed and pooled and cells were cultured with IPC-2 as shown. Supernatants were analyzed for cytokine production.

‡ Each number represents the arithmetic mean of values from triplicate cultures plus and minus the SEM, shown in parentheses.

Example 2Subcutaneous and intravenous administration of peptide Y induces T cell tolerance in mice

Four groups of five BDF1 (C57BL/6J x DBA/2J) mice (females, 6-8 weeks of age), were injected either subcutaneously in a single dorsal site between the forelimbs or intravenously through one of the tail veins. For each injection site on day 0 and day 5, one group of animals was injected with 300 µg peptide Y in Phosphate Buffered Saline (PBS) and the other was injected with PBS alone. On day 10, each animal was challenged with 100 µg peptide Y in 200 µl Complete Freuds adjuvant (CFA) in four sites, two subcutaneous sites at the base of the tail and two subcutaneous sites on the thigh. The animals were sacrificed by cervical dislocation on day 20 and inguinal and popliteal nodes were removed and placed in rinsing buffer, cold RPMI 1640 containing 1% Fetal Bovine Serum (FBS). The nodes were rinsed with rinsing buffer and forced through a fine stainless steel mesh, using a glass pestal to suspend them in rinsing buffer. The suspended cells were rinsed two times by centrifugation at 1500 rpm for 10 minutes and resuspended with rinsing buffer. An aliquot of suspended cells from each animal was counted on a Coulter Counter Model ZB.

The cells (4×10^6 /ml) were incubated in culture media (RPMI media containing 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol) and with either 150 µg/ml peptide Y (Fig. 4) or alone. The 0.2 ml cultures were done in triplicate in flat bottom 96 well plates (Costar) at 37°C and 5% CO₂. After 24 hours, 50 µl of the media from each well was

placed in separate round bottom 96 well plates (Costar) and was frozen overnight at -20°C to eliminate carryover of live cells. The supernatants were tested after thawing for their ability to support the growth of CTLL-2, an IL-2 dependent T cell clone (ATCC TIB#214). CTLL-2 in log phase growth were rinsed 2 times by centrifugation at 1000 rpm for 10 minutes, aspiration of the media, and resuspension of the pellet with culture media. CTLL were added to the warmed culture supernatants (5×10^3 CTLL cells/well) and the IL-2 assay was incubated at 37°C and 5% CO_2 . After 24 hours, $1 \mu\text{Ci/ml}$ ^3H -thymidine was added in $50 \mu\text{l/well}$ and the CTLL cells were incubated an additional 4-6 hours, then they were harvested with a Tom-Tec 96-well cell harvester. The ^3H -incorporation in each well was counted by a Betaplate Model 1205 scintillation counter. Background counts were not subtracted.

Each bar on Figure 4 represents the arithmetic mean of triplicate in vivo cultures from one mouse. After in vivo challenge with peptide Y in CFA, lymph node cells from mice which were administered saline by either route specifically responded to challenge with peptide Y in vitro, as shown by IL-2 production (white bars). In contrast, draining lymph node cells isolated from mice who were administered peptide Y intravenously and subcutaneously exhibited a decreased specific IL-2 secretion following challenge with peptide Y in vivo. (dark bars). The results of this experiment demonstrate that subcutaneous and intravenous administration of peptide Y results in T cell tolerization as shown by the decrease in antigen specific production of IL-2.

Example 3Subcutaneous administration of peptide Y dose response for induction of T cell tolerance in mice

In another experiment, BDF₁ mice were tolerized
05 with various doses of peptide X and peptide Y prior
to challenge with the peptide in order to determine
the lowest dose necessary for the induction of
tolerance. Seven groups of mice, each group
containing three BDF₁ mice, were injected
10 subcutaneously with 0.2 ml PBS containing from 0 -
500 µg/ml peptide Y (Figure 5) or peptide X (Figure
6). Each group of animals was injected at a single
dorsal site under the skin between the forelimbs on
both day 0 and on day 5. On day 10 all of the
15 animals received a challenge injection of 100 µg
peptide Y or peptide X in 200 µl CFA as described
above. On day 20 the animals were sacrificed and
draining lymph nodes were removed as described
above. The lymph node cells were suspended, rinsed,
20 and cultured as above with or without 150 µg/ml of
peptide Y or peptide X, as appropriate. The
supernatants from the cultures were assayed for IL-2
as described above.

Each bar in Figures 5 and 6 represents the
25 average of triplicate cultures of pooled lymph node
cells from three mice. The baseline for control
cultures (i.e., cells cultured without peptide X or
peptide Y) for the IL-2 assay were approximately 1500
cpm (Figure 5) and approximately 300 cpm (Figure 6).
30 The dose of peptide per injection needed to
completely decrease peptide-specific IL-2 production
is between 4 and 20 µg/dose for these experiments
using two injections (day 0 and day 5). The results

demonstrate that BDF₁ strain mice can be tolerized at the T cell level with small doses of peptide X or peptide Y.

Example 4

05 Subcutaneous administration of a combination of peptide X and peptide Y induces T cell tolerance in mice

In order to determine whether peptide X and peptide Y can induce tolerance when injected in
10 combination, B6CBAF₁ mice (C57BL/6J x CBA/J) were tolerized and challenged with a mixture of the two peptides. B6CBAF₁ mice respond well to both peptide X and peptide Y, as measured by peptide-specific IL-2 production from lymph node cells isolated from mice
15 who were challenged with either peptide. In this experiment, one group of five B6CBAF₁ mice was injected with 300 µg/dose each of peptide X and of peptide Y. The animals were injected at a single dorsal site under the skin between the forelimbs on
20 days 0 and 5. On day 10 the animals received a challenge dose of 100 mg each peptide X and peptide Y in 270 µl total CFA in above location. On day 20 the animals were sacrificed and draining lymph nodes were removed as described above. The lymph node cells
25 were isolated, suspended, rinsed, and cultured as above with or without 150 µg/ml of peptide Y, peptide X; or a combination of peptide X and peptide Y. The supernatants from the cultures were assayed for IL-2 as described above.

30 Each bar in Figure 7 represents the arithmetic average of cpm values of triplicate cultures of pooled draining lymph nodes of five animals. Tolerization of animals with a combination of peptide

X and peptide Y results in T cell tolerance in these animals against both of the peptides, presented in vitro either separately or together.

Example 5

05 Peptide Y specific T cell response in mice
preimmunized with TRFP is tolerized by peptide Y when
administered subcutaneously

In order to demonstrate that subcutaneous administration of peptide Y can induce T cell
10 tolerance in primed mice, mice were immunized with whole protein, TRFP, so that there would be T cell response to peptide Y derived from the TRFP protein. Peptide Y in the native protein structure is obscured by the protein conformation. Thus, following TRFP
15 administration, the mice do not produce antibody specific for peptide Y.

BDF₁ mice were each immunized with 100 µg TRFP in 200 µl IFA (Incomplete Freund's Adjuvant) in a single site intraperitoneally. After four months,
20 the mice were bled and grouped according to their anti-TRFP IgG antibody levels as follows. The mice were warmed with an infra-red lamp and bled through one of the tail veins, by nicking the vein with a razor blade (Gillette Platinum Plus). The blood was
25 allowed to drip into a serum separating tube (Microtainer, Becton-Dickenson) and allowed to clot. The sera was then separated from the clot by centrifugation at 13,000 rpm. The sera was stored at -20° until use. The sera was assayed for
30 TRFP-specific IgG by ELISA. For the IgG assay, immunoaffinity purified TRFP was coated onto Nunc Polysorp 96-well plates by incubation of 50 µl/well of 2µg/ml TRFP in PBS. Unless mentioned, each

incubation step in this protocol was performed for 1 hour at 37°C. After rinsing with PBS, the wells were incubated with 0.5% gelatin in PBS to block excess binding to the plate. The plates were rinsed with
05 PBS containing 0.05% Tween 20. Sera were diluted 1/500 in PBS and incubated in triplicate on the plates. After rinsing, the bound mouse antibody was detected by incubation with biotinylated goat anti-mouse IgG (Southern Biotechnology Associates)
10 diluted 1/10000 in PBS. The plates were rinsed and streptavidin conjugated to horse radish peroxidase (Southern Bio.Ass.) diluted 1/10000 was added for 20 minutes and incubated at room temperature. TMB peroxidase substrate (Kirkegaard and Perry) was used
15 according to directions supplied. The resulting O.D. values were read by an ELISA reader (Bio-Tek modal #310) at 450 nm to quantitate the complexes bound to TRFP protein on the plates. The mice were matched for O.D. of the developed ELISA wells using the 1/500
20 dilution of each sera and separated into two matched groups of three animals. The range of the values for the TRFP specific IgG of 1/500 dilution sera from these animals was between 1.7 and 2.2.

To determine whether T cells from these TRFP
25 primed mice respond to peptide Y, three TRFP primed, anti-TRFP antibody positive mice (not in the IgG matched groups above, but with similar IgG levels) were sacrificed by cervical dislocation and the spleens were removed and placed in rinsing buffer.
30 Spleens were used because intraperitoneal injection leads to strong T cell responses in the spleen. The spleens were suspended and rinsed as detailed above for lymph node cells. The cells were cultured as above with 150 µg/ml peptide Y or with media alone.

After 24 hours the supernatants were removed and tested for the presence of IL-2 as previously described. The peptide Y specific IL-2 production shows that these mice responded to peptide Y 4 months
05 after TRFP priming in IFA (Figure 8).

In addition, TRFP IgG matched groups of mice were tolerized with peptide Y. The tolerance of these animals to peptide Y was then shown by their failure to specifically secrete IL-2 in vitro
10 following an in vivo challenge with peptide Y. The IgG matched groups were tolerized and challenged with peptide Y as follows. One of the two IgG matched groups was injected subcutaneously on days 0 and 5 (day 109 and day 114 after TRFP priming) with 0.2 ml
15 PBS containing 300 µg peptide Y. The other group was injected with PBS only. Both groups were injected at a single dorsal site between the forelimbs. On day 10 (day 119 after TRFP priming) both groups of animals were challenged with 100 µg peptide Y as
20 described above. On day 20 (day 129 after TRFP priming) the animals were sacrificed and the draining lymph nodes were removed as described above. The lymph nodes were suspended, rinsed, and cultured as described above with 150 µg/ml peptide Y (Figure 9,
25 left panel) or with media alone. After 24 hours, the supernatants of the cultures were tested for IL-2 as described above.

Each bar in the left panel of Figure 9 represents the mean of triplicate cultures of one
30 mouse. This experiment demonstrates that T cells specific for peptide Y can be tolerized in animals which have been preimmunized with TRFP and demonstrate a peptide Y specific T cell response at the time of tolerization.

Example 6

sc tolerization with peptide Y decreases the peptide specific antibody response in animals preimmunized with TRFP

05 The ability of mice to mount an antibody response to peptide Y can be used as an assay for peptide Y specific T helper cell activity. As shown in Example 5, immunization of BDF1 mice with TRFP generates antibodies specific for whole TRFP, with no
10 detectable antibodies specific for peptide Y. The two matched groups of mice described above were bled at the time of sacrifice (ten days after peptide Y/CFA challenge). The serum was separated as previously described. The sera were assayed both for
15 IgG antibody binding specifically to peptide Y. For the IgG assay, immunoaffinity purified peptide Y was coated onto Nunc Polysorp 96-well plates by incubation of 50µl/well of 20µg/ml peptide Y in PBS. Each incubation step in this protocol lasts 1 hour at
20 37°. The wells were incubated with gelatin in PBS for blocking excess binding to the plate. Sera were diluted in PBS in a range from 1/300 to 1/8100 and incubated in triplicate on the plates. After rinsing, the bound mouse antibody was detected by
25 incubation with biotinylated goat anti-mouse IgG (Southern Biotechnology Associates). Streptavidin conjugated to horse radish peroxidase (Southern Biotechnology Associates) was added to detect antigen bound biotinylated antibody-complexes. TMB
30 peroxidase substrate (Kirkegaard and Perry) was used according to directions supplied and the resulting O.D. values were read by an ELISA reader (Bio-Tek model #310) at 450 nm to quantitate the horse radish peroxidase-containing complexes bound to peptide Y on

the plates. The O.D. 450 of the ELISA wells of the sera at 1/900 are shown in Figure 9. The data from the other sera dilutions were consistent with the data from the 1/900 sera dilution. The peptide Y
05 tolerized animals made little antibody specific for peptide Y in the 10 days after peptide U/CFA challenge, much less than the animals that were not tolerized. These results suggest that the tolerization of TRFP preimmunized animals with
10 peptide Y reduces peptide Y specific T cell helper activity for antibody production.

The sera were assayed for the presence of peptide Y specific IgM, IgE, and IgG isotypes. The ELISA used to detect antigen specific binding of
15 IgG1, IgG2a, IgG2b, IgG3 and IgM isotypes were similar to the IgG assay described above with the only difference being the biotinylated anti-immunoglobulin used to detect the bound isotype. These assays employed biotinylated goat
20 anti-IgG1 (#1070-08), Southern Biotechnology Associates), anti-IgG2a (#1080-08, SBA), anti-IgG2b (1090-08, SBA) anti-IgG2 (1100-08, SBA), and anti-IgM (1020-08, SBA). Antigens bound IgE was detected similarly, but using biotinylated EM95, a rat
25 monoclonal antibody specific for mouse IgE. (Baniyash M, Eshhar Z: Inhibition of IgE binding to mast cells and basophils by monoclonal antibodies to murine IgE. Eur J. Immunol. 14:799, 1984). Biotinylated goat anti-rat Ig was used as an added
30 signal amplification step in the IgE ELISA. No binding above background was detected in assays for peptide Y specific IgG2a, IgG3, or IgE. IgG1 was the major isotype seen in the animals which were injected with saline only, with a titer of 1/2700 for all

three animals. Of the peptide Y tolerized animals, two had an IgG1 titer of 1/300 and one had no binding above background. The titers for IgM and IgG2b were similarly effected. The sera from all of the
05 tolerized animals had no IgG2b binding above background while each of the saline control animals had serum titers of 1/300. The peptide Y tolerized animals all had serum IgM titers of 1/300 while the saline controls all had serum IgM titers of 1/900.
10 Thus the sc peptide injected in saline decreased the antibody response in these primed animals without changing the isotype distribution of the anti-peptide antibody.

Example 7

15 Administration of peptide Y to mice preimmunized with TRFP has no effect on antibody specific for TRFP

The animals described in Example 6 which were tolerized to peptide Y had a preexisting antibody response to whole TRFP protein. The anti-TRFP IgG
20 response of these animals before and after the "tolerization" injection (i.e., peptide Y in PBS vs. PBS alone) and the challenge injection (i.e., peptide Y in CFA) was compared. This comparison allows a determination of whether this T cell tolerization has
25 an immediate effect on the TRFP specific IgG levels. The anti-TRFP antibody assay was followed as detailed above. The mouse sera was diluted from 1/500 to 1/13,500 for this assay. The data from the 1/4500 sera dilution is shown in Table 2. Each sera was
30 assayed in duplicate wells and the values were averaged. The background in this assay was about 0.1. The data show that there was no significant change in the quantity of antibody specific for TRFP

in any of these mice in this time frame. Thus, tolerization of the T cells specific for peptide Y had no effect on the anti-TRFP antibody in the 20 days following tolerization.

05

Table 2

ELISA analysis of specific IgG binding of mouse sera to TRFP

	ELISA O.D.-sera sampled before "tolerization" and challenge	ELISA O.D.-sera sampled after "tolerization and challenge	
10			
	"tolerization" with PBS only		
	- mouse #1	0.842	0.782
15	- mouse #2	0.580	0.503
	- mouse #3	0.725	0.693
	"tolerization" with peptide Y in PBS		
	- mouse #1	0.670	0.492
20	- mouse #2	0.687	0.654
	- mouse #3	0.686	0.585

The values given are averages of duplicate wells of 1/4500 dilutions of the sera.

Example 8Subcutaneous administration of peptide Y induces tolerance in IL-2 and IL-4 producing T cells in mice primed with TRFP

05 There are several phenotypic classes of T helper cells which can be differentiated by their production of lymphokines. Th₁ cells produce IL-2 and γ-interferon, as well as other lymphokines. Th₂ cells produce IL-4 and other lymphokines. IL-2
10 production is easily measured from primary in vitro lymph node cultures through the IL-2 dependent CTLL-2 cell line. Similarly, IL-4 can be measured with cultures of the IL-4 dependent CT4S cells (provided by W. Paul). However, production of IL-4 from
15 primary in vitro culture is low and not reproducible. In order to determine whether tolerization with peptide Y effects both types of T helper cells, we pooled lymph node cells from the two groups of mice (described previously) that were
20 tolerized, challenged, and assayed for T cell tolerance. The cells (2×10^7 total) were cultured in 5 ml culture media containing 30 μg/ml peptide Y. After 21 days incubation at 37°C and 5% CO₂, the cells were rinsed two times with culture media and
25 replated at 4×10^6 cells/ml in triplicate 0.2ml wells with or without 150 μg/ml peptide Y for both the IL-2 production assay and the IL-4 production assay. Samples (50 μl each) of the supernatants for the IL-2 assay were removed at 24 hours and assayed
30 as detailed above. Samples for the IL-4 assay were removed at 48 hours and stored at 4°C until use (2 days minimum). The IL-4 content was assayed by the ability of the supernatants to support the growth of the CT4S line. CT4S cells (1×10^4 /well) were added

to the warmed culture supernatants and incubated 40 hours at 37°C and 5% CO₂. ³H-thymidine (1 µCi/50 ml culture media/well) was added for an additional 8 hours of culture to quantitate CT4S growth through incorporation of the thymidine into DNA. The cells were harvested after freezing the plates to dislodge the slightly adherent CT4S cells. The plates were harvested and counted identically to the IL-2 assays detailed above.

For both the secondary IL-2 and the secondary IL-4 assays (Figure 10), the average number of counts from the triplicate 150 µg/ml peptide Y wells was divided by the average number of counts from the wells without peptide Y to determine a stimulation index. This was necessary because the no antigen background in the secondary cultures is more variable than in primary cultures. The peptide tolerization decreased the peptide specific production of both IL-2 and IL-4 in these secondary in vitro cultures. These data suggest that tolerization with peptide Y decreases the antigen specific production of both IL-2 and IL-4. The effect indicates a tolerogenic effect by administration of peptide Y on both classes of T helper cells.

25 Example 9

Subcutaneous administration of peptide X and peptide Y decreases T cell response to recombinant chain 1 of TRFP

Four groups of B6CBAF₁ mice were "tolerized" by subcutaneous injection with either PBS alone or PBS containing recombinant chain 1 of TRFP; PBS containing peptide X and peptide Y; or PBS containing peptides Fel 3-1, peptide Y, peptide C, and peptide

D. The mice were injected with 150 µg/dose of either the TRFP protein or each of the peptides (days 0 and 5). The PBS alone and the PBS/peptides were injected in 300 µl/dose. The PBS/chain 1 of the TRFP protein
05 was injected in 100 µl/dose. All tolerizing doses were at a single site under the dorsal skin between the forelimbs. On day 10 all of the animals received a challenge injection of 100 µg recombinant chain 1 of TRFP in 200 µl CFA as described above. On day 20
10 the animals were sacrificed and draining lymph nodes were removed as described above. The lymph node cells were suspended, rinsed, and cultured as above with or without 150 µg/ml recombinant chain 1 of TRFP. The lymph node cell cultures in this
15 experiment were each 0.1 ml. The supernatants from the cultures were assayed for IL-2 as described above.

Each bar in Figure 11 represents the average of IL-2 produced by triplicate cultures of lymph node cells from five individual mice. IL-2 assays from
20 cultures of these lymph node cells without peptide was approximately 500 cpm. The results show that tolerization with peptide X and peptide Y and tolerization with chain 1 of TRFP is effective in decreasing IL-2 production specific for chain 1 of
25 TRFP after in vivo challenge with the chain 1 protein TRFP. The results indicate that tolerization with two peptides from chain 1 (peptide X and peptide Y) are as effective as tolerization with four peptides from chain 1 (Fel 3-1, peptide Y, peptide C and
30 peptide D) or as effective as tolerization with TRFP chain 1 itself.

Example 10IgE Binding Studies With peptide X and peptide Y

The objective of these studies was to analyze the extent of human IgE binding to peptide X and peptide Y derived from the TRFP chain 1 sequence. An ELISA assay had been previously performed to quantitate IgE binding to TRFP with cat allergic patient plasma as the source of IgE. This assay format was then extended to analyze the potential for anti-TRFP IgE to bind peptides X and Y. These assays also included equal mixtures of the two peptides and each peptide separately conjugated to HSA (human serum albumin). Dot blot assays on some of these antigens were also run as an alternative means of detecting IgE binding. The clinical implications would be that if these peptides bind a significant portion of anti-TRFP IgE, adverse allergic reactions would be more likely during treatment of an allergic individual.

20 ELISA

The basic format of these ELISA assays was a direct binding assay wherein the antigen was coated onto the wells of a 96 well microtitre dish and then antibodies in solution are added and assayed for the binding capacity. The protocol for these assays was as follows:

Corning assay plates (#25882-96) were coated with 10 µg/ml of each coating antigen; affinity purified TRFP, peptide X, or peptide Y, peptide X and peptide Y mixed, peptide X/HSA, or peptide Y/HSA in PBS (phosphate buffered saline), at 50 µg/well and incubated overnight at 4°C. The peptides were conjugated to HSA (human serum albumin) by two

different methods based on their chemical structure. The conjugation reaction with peptide X used the reagents and protocol of the Pierce Imject Immungen EDC conjugation kit (Pierce, Rockland, IL). Peptide
05 Y was conjugated to HSA through its cysteine residue using the sulfo-SMCC reagent (Pierce). Both conjugates were filter purified and dialyzed against PBS. In the ELISA assay, the unbound coating
10 antigens were removed and the wells were blocked with 0.5% gelatin in PBS, 200 µl/well for 2 hours at room temperature. The antibody solution was a pool of plasma samples from 20 patients that were skin test
15 positive for commercial cat extract which was serially diluted with PBS-Tween 20 (PBS with 0.05% nonionic detergent Tween-Sigma, St. Louis MO) and 100µl/well was added and incubated overnight at 4°C (plasma dilutions are tested in duplicate). This
20 plasma pool had been depleted of most of the IgG antibodies by extraction with Protein G-Agarose (GammaBind G-Agarose, Genex Corp. Gaithersburg, MD). The second antibody (biotinylated goat anti-Human IgE, 1:1000 Kirkegaard & Perry Laboratories Inc. Gaithersburg, MD), was added at 100µl/well for one
25 hour at room temperature. This solution was removed and streptavidin-HRPO, 1:1000, (Southern Biotechnology Associates, Inc., Birmingham, AL) was then added at 100µl/well for one hour at room temperature (all wells were washed three times with
30 PBS-Tween between each incubation step). TMB Membrane Peroxidase Substrate system (Kirkegaard & Perry Laboratories) was freshly mixed, and added at 100µl/well. The color was allowed to develop for 2-5 minutes. The reaction was stopped by the addition of 100µl/well of 1 M phosphoric acid. Plates are read

on a Microplate EL 310 Autoreader (Biotek Instruments, Winooski, VT) with a 450nm filter. The absorbance levels of duplicate wells were averaged. The graphed results (log of the dilution vs
05 absorbance) of the ELISA assays are shown in Fig. 12.

Figure 12 is the graphic representation of ELISA assays for TRFP, peptide X and peptide Y IgE binding. Because IgE antibody is the least abundant antibody in human plasma the first dilution, 1:2 in
10 PBS, shows an absorbance reading of 0.65 for TRFP binding. Although clearly positive for binding, this is still a low initial absorbance reading. Positive binding is defined as absorbance readings that are greater than or equal to two times the background
15 level. Some individual patients have much higher levels of anti-TRFP IgE which is reflected in the higher absorbance readings (data not shown). This pool of IgE depleted plasma represents an average of all the different patients anti-TRFP IgE. This assay
20 can accurately detect the relative quantity of anti-TRFP IgE reactive with the coated TRFP antigens. The two peptide antigens, peptide X and peptide Y, do not demonstrate any specific IgE binding at any of the dilutions examined. The
25 negative control for these binding assays is the level of binding to wells which have had no antigen coated on them. For the positive control for the presence of peptide coated on the wells was an ELISA using anti-peptide antisera against either peptide X
30 or peptide Y was used.

Table 3 shows the percent positive binding as summarized data from different numbers of cat allergic patients. A smaller set of patients was examined for IgE binding to the mix of peptides and

the peptides conjugated to HSA. Again the positive control for the presence of peptides or peptide conjugates was binding of anti-peptide antisera (now shown).

05

Table 3

Summary of Analysis of Direct IgE Recognition of
peptide X and peptide Y

ELISA*

	Antigens tested:	Positive Binding	N
10	TRFP	100%	12
	Peptide X	0%	12
	Peptide Y	0%	12
	Peptide X+IPC-2	0%	4
	Peptide X/HSA	0%	3
15	Peptide Y/HSA	0%	3

Dot Blot

	Antigens tested:	Positive Binding	N
	TRFP	100%	8
	Peptide X	0%	8
20	Peptide Y	0%	8

IgE binding studies using a similar protocol as described above were carried out using peptides Fel 8-1, Fel 31-1, Fel 31-2, Fel 31-4, and Fel 31-3. None of these peptides showed IgE binding.

Dot Blots:

The dot blot assay is similar to ELISA in that is a direct binding assay with the antigen bound to a solid phase matrix. In the case of the dot blot
05 assay the matrix is nitrocellulose paper and results are based on autoradiography with ^{125}I -Streptavidin binding to a biotin labeled second antibody.

Dot blot protocol: Antigen; TRFP, peptide X and peptide Y, were coated on 0.1 μm nitrocellulose sheet
10 using 96-well dot blot manifold (Biorad, Richmond, CA) for 45 min. at room temperature in 100 μl sample volumes. TRFP was coated at 2, 0.2 and 0.02 $\mu\text{g}/\text{dot}$ and the peptides at 10, 1 and 0.1 $\mu\text{g}/\text{dot}$. Wells were then washed with 200 μl PBS. After equilibration of
15 blots with Tween solution, (Tris-HCL; pH 7.5 0.025M NaCl 0.17M Tween-20 0.5%) all sections were blocked with 1% nonfat dry milk, 1% fetal calf serum/Tween solution for 1-1.5 hour. Patient plasma was used at 10% (v/v) in 1% milk/Tween solution and preabsorbed
20 on blank strips of nitrocellulose for 1 hour. After preabsorption, the diluted patient plasma sample was incubated with the antigen bearing dot blot overnight at room temperature (RT). Nitrocellulose dot blot was incubated in biotinylated goat anti-human IgE
25 (1:2500) in Tween solution for 2 hours RT. After washing, the sections were incubated in 1 μCi ^{125}I -Streptavidin, 1 hour. Following removal of unbound label by extensive washing, the blots were exposed to film for 16 hours.

30 The summarized results of the dot blot assays are shown in Table 3. At all concentrations the peptide antigen dots were negative for specific IgE binding and with this patient set all were positive for TRFP binding.

Conclusion:

By using the direct binding ELISA method we have clearly demonstrated IgE binding to affinity purified TRFP. With this same assay we detect no specific
05 binding of cat allergic IgE to peptide X and peptide Y. For further demonstration of lack of peptide binding, a mixture of the two peptides as the coating antigens was examined. This mixture was negative for detectable, specific IgE binding as were the
10 individual peptides which has been conjugated to human serum albumin. The rationale for including these forms was to check that the lack of binding was not just due to some conformation of the peptide as it is directly bound to the plate. These results are
15 correlated with those from the dot blot assay wherein a distinctly different matrix is used that includes a different end-point readout (i.e., autoradiography versus enzymatic color development). No direct IgE binding to Fel 8-3 and Fel 30-4 was detected.

20 Example 11Histamine Release Analysis Comparing TRFP to peptide X and peptide Y

The objective of the histamine release analysis was to directly assay the effects of peptides or TRFP
25 in an in vitro allergic response system. The release of histamine through IgE recognition and IgE receptor crosslinking on live cells directly assays the allergic potential of a protein antigen. The aim of these studies was to compare this allergenic
30 potential between the known allergen TRFP and peptide X and peptide Y.

The histamine release assay used for these studies is based on the detection of an acylated

derivative of histamine using a specific monoclonal antibody (Morel, A.M. and Delaage, M.A.; 1988, J. Allergy Clin. Immunol. 82:646-654.) This assay was performed as two separate protocols: 1) the release
05 of histamine from basophils present in heparinized whole blood in the presence of different concentrations of antigens and 2) the actual assaying of histamine present in the supernates of the release reactions following cell removal by centrifugation.
10 The reagents for this histamine radioimmunoassay are supplied commercially as a kit by Amac Inc. (Westbrook, ME).

Whole blood was drawn from cat allergic patients using heparinized syringes or vacutainers. The
15 antigens, affinity purified TRFP, peptide X and peptide Y, were diluted to 2x concentration in PACM (PIPES buffer 25mM, NaCl 110mM, KCl 5.0mM, human serum albumin 0.003%, CaCl₂ 5mM, MgCl₂ 2mM, pH 7.3) buffer with 0.2 ml in each 1.5 ml polypropylene
20 tube. The same volume of blood, 0.25 mls, was added to each tube and the reactions were started by inversion. The buffer control consisted of whole blood with just buffer and no added antigen. The release reactions were then carried out at 37°C for
25 30 minutes. After this incubation the tubes were centrifuged at 1500 RPM for 3 minutes and the supernates were carefully removed. It was important to spin gently so that there would be no cell lysis giving misleading results. For the total histamine
30 release values a 100 µl blood sample was boiled for 3 minutes in a total volume of 1 ml with PACM buffer. At this point the supernates were either frozen at -20°C for later analysis or processed immediately.

For the RIA analysis, a 50 μ l aliquot of the release supernatant was diluted 1:4 with Histamine release buffer (supplied with the kit from Amac Inc.) Then 100 μ l of each diluted supernatant was
05 added to the tubes containing the acylation reagent (a lyophilized powder). Immediately after the addition of each supernatant, 50 μ l of acylation buffer was added and the tube mixed by vortexing until the reactants were completely dissolved. The
10 acylation reactions were allowed to go to completion at R.T. for \geq 30 minutes. A set of histamine standards was supplied with the kit and these all were processed by the acylation reaction (i.e., 100 μ l of each standard and 50 μ l of acylation buffer in
15 each acylation reaction tube.) Then 50 μ l of each acylation reaction was added to the bottom of a monoclonal antibody coated 12x75 mM plastic tube. This monoclonal antibody recognizes the acylated histamine derivative as its epitope. Finally, 500
20 μ ls of ^{123}I labelled tracer was added to each tube on ice and the binding reaction was carried out overnight at 4°C (\geq 18 hrs). The binding reactions were stopped by aspiration of the solutions from each tube. Then they were counted on a gamma counter
25 (Cobra 5005, Beckman Inc.) for two minutes/tube.

Following the quantitation of ^{125}I label/tube the standard curve is generated from the histamine standard counts and plotted on semi-log paper. Because this is a competition assay, with labelled
30 ^{125}I tracer competing with the standards or unknowns for cell supernatants, the lower the recorded counts the greater the amount of cold acylated histamine in the binding assay. The data points were then generated by reading off the values on the plotted.

standard curve of counts versus histamine concentration (in nM).

This type of analysis for histamine is a very sensitive, accurate method of measurement.

05 Figure 13 shows the summarized graphed results of eight individual cat allergic patients comparing histamine release following culture with various concentrations of affinity purified TRFP, peptide X and peptide Y. The graph shows the concentration of
10 the various antigens in nMoles versus the percent total histamine released for each patient. The graph depicts the average percent release for each concentration point and the standard error of the mean are shown with error bars. Although only one
15 reaction/patient is performed with PACM buffer alone it is represented as a line for comparison with antigen release levels.

The results are represented as percent total histamine released because of the great variability
20 between patients in overall histamine levels. The source of this variability relates to the number of basophils per unit blood from patient to patient and the variation in quantity of histamine per basophil. There are only six (5-fold) concentration points for
25 the TRFP peptide antigens emphasizing the higher end of the concentration curve. The TRFP concentrations are eight five-fold dilutions with three lower concentrations used for TRFP to get the full response range.

30 The histamine release profile shown in Figure 13 demonstrates that TRFP, at all but the lowest concentration, gives a clear release signal. However, there is the appearance of a plateau of release at lower concentrations with increasing

release at higher antigen concentrations. The typical histamine release profile with a single, purified allergen should theoretically show a bell-shaped curve with lower histamine released at higher concentrations due to lack of crosslinking (i.e., each IgE molecule bound to a cell surface receptor binds a separate allergen molecule). This was not the case in the current experiments with TRFP. There is no discernible histamine release to either peptide X or peptide Y.

Example 12

Reassociation of TRFP Recombinant Chains 1 + 2

The two recombinant chains of TRFP were reassociated in vitro as a means of increasing IgE reactivity and generate a reagent that more closely resembles the native Fel d I which can be used in its place, especially for those assays that require a large quantity of material. The reassociated TRFP may be useful as a diagnostic reagent.

The protocol used for the reassociation required 1 mg of each recombinant chain (rchain) mixed under the following conditions;

Reaction conditions

- 1) 1 mg of rchain 1&2, 2) 6.56M Urea (final conc.)
- 3) 50 mM DTT 4) 0.2X phosphate buffered saline (PBS)

The final volume was 5 mLs and was heated to 55°C for 3 minutes with shaking and then 5 mLs H₂O was added and the mix was allowed to cool to room temp. This was followed by extensive dialysis against 1X PBS at room temp using a membrane with a molecular weight cutoff of 3,500.

The reassociated TRFP was analyzed by a direct ELISA followed by a depletion ELISA. The protocol for the ELISA is as follows: Microtiter plates were coated with 5.0µg/mL of coating antigen (Fel d I,
05 recombinant TRFP (rTRFP) chain 1, rTRFP chain 2, a mix of rTRFP chain 1+2, or reassociated rTRFP chain 1+2) in PBS at 100µL/well and incubated overnight at 4°C. The plates were washed three times between each step with PBS-T (Phosphate buffered saline + 0.05%
10 Tween 20). The unbound antigen was removed and the plate blocked with 300µL/well of 1% bovine serum albumin (BSA) in PBS-T for one hour at room temperature. All subsequent reagents were added at 100µL/well. For direct ELISA, serially diluted human
15 plasma was added to duplicate wells and incubated overnight at 4°C. This was followed by biotinylated goat anti-human IgE (1:1,000) for one hour at room temperature, then streptavidin-HRPO (1:10,000) for one hour at room temperature. TMB substrate and H₂O₂
20 were freshly mixed and added; the color was allowed to develop for 2-5 minutes. The reaction was stopped by the addition of 1M phosphoric acid. The plates were read on a Dynatech plate reader at 450nm and the absorbances of duplicate wells were averaged. For
25 the depletion ELISA, patient plasma was pre-incubated on antigen or PBS coated wells, plasma collected and re-incubated on freshly coated wells. The ELISA was then performed as outlined above.

The results of the direct ELISA shown in Fig. 21
30 demonstrated that the reassociated TRFP had similar binding properties to native Fel d I, the two separate recombinant TRFP chain and the mixture of rchain 1 and rchain 2. As shown in Fig. 22, the depletion ELISA demonstrated that the reassociated

material had properties more closely matching Fel d I than either the two chains separately or mixed. Pre-absorption on Fel d I showed the greatest depletion of human IgE against Fel d I (lowest
05 binding curve); pre-absorption on the reassociated chains exhibited significantly greater reduction of Fel d I specific IgE (second lowest binding curve) than pre-absorption on a mix of the chains or the individual chain. It appeared that the reassociated
10 recombinant chains either have more epitopes for IgE binding (than a mixture of rchain 1 and rchain 2) and/or that the affinity of binding to the epitopes has been increased.

EXEMPLIFICATION B

15 In another set of experiments, following a different methodology, T-cell proliferation, IL-2 production and IL-4 production for four cat allergic patients was studied. These experiments examined the ability of peptides comprising epitopes of TRFP to
20 induce in vitro proliferation of T cells from cat allergic patients and whether this proliferation can be linked to the synthesis of the cytokines, interleukin 2 (IL-2) and interleukin 4 (IL-4).

In addition the peptides shown in Tables 4 and
25 5, peptides Fel-32, Fel-33, Fel-34, Fel-35, Fel-36, Fel-37, Fel-38, Fel-38-1, Fel-39, and Fel-39-1 as shown in Figure 14 were included in these experiments.

Table 4

Response of Cat Allergic Patients to Chain 1 TRFP Peptides

<u>Peptide</u>	<u>Amino acid</u>	<u>PI¹</u>	<u>SI²</u>	<u>N³</u>
Fel 1 ⁴	1 - 17, 3T	392	5.6	121
1-2	1 - 17	311	7.4	83
1-3	4 - 17	422	6.2	26
1-4	6 - 17	816	9.6	32
1-5	8 - 17	286	5.2	25
1-6	10 - 17	312	5.2	26
Fel 2	9 - 25	416	7.3	30
Fel 3 ⁶	18 - 33, 31P, 32D	674	9.5	123
3-1	18 - 33	638	9.4	40
3-10	18 - 31	504	6.9	28
3-11	18 - 30	863	10.4	12
3-15	18 - 29	1040	10.4	13
3-13	18 - 28	690	11.5	14
3-14	18 - 27	260	6.2	10
Fel 8	1 - 30	1393	18.1	86
8-1	5 - 33	1374	15.1	47
8-2	6 - 33	1353	15.2	47
8-3	7 - 33	1437	16.9	47
Fel 14	18 - 43	1054	13.7	125
14-1	23 - 36	871	9.9	88
14-3	25 - 36	621	6.9	90
14-4	26 - 36	474	6.0	79
14-5	27 - 36	286	5.4	53
14-2	29 - 42	336	4.6	60
Fel 4	37 - 55	601	7.7	120
4-1	37 - 52	822	9.9	20
4-2	37 - 49	378	6.2	15
4-3	37 - 46	185	3.7	13
Fel 30-1	25 - 49	268	5.6	23
30-2	25 - 48	248	4.2	22
30-3	25 - 47	230	4.8	23
30-4	29 - 55	1079	11.6	44
30-5	29 - 54	792	11.0	43
30-6	29 - 53	415	6.2	43
30-7	26 - 55	339	5.3	14
30-8	28 - 55	262	4.1	14

Fel 15	44 - 60	440	11.0	60
Fel 23	51 - 66	343	6.6	63
Fel 21 ⁵	56 - 70, 70R	360	5.8	66

PI:1 Average SI of all responding patients tested multiplied by the percent of those patients with a positive response

SI:2 Average of the cpm of T cell and antigen presenting cell proliferation to the antigen divided by cpm of T cells and antigen presenting cells alone from responding patients. An SI of ≥ 2.5 is considered positive.

N:3 Number of patients tested.

4: Amino acid 3 changed to T.

5: Amino acid 70 changed to R.

6: Amino acids 31 and 32 changed to P and D, respectively.

Table 5

Response of Cat Allergic Patients to Chain 2
TRFP peptide

<u>Peptide</u>	<u>Amino acid</u>	<u>PI</u>	<u>SI</u>	<u>N</u>
Fel 16	1 - 22	283	5.9	52
Fel 17	12 - 33	421	6.1	114
Fel 32-1	12 - 24	442	6.6	21
32-2	14 - 24	424	5.3	20
32-3	16 - 24	270	3.7	22
Fel 18	23 - 48	466	6.3	99
Fel 33-1	26 - 36	340	5.4	63
33-2	26 - 38	210	4.2	50
33-3	26 - 40	235	5.0	47
Fel 31-1	14 - 40	733	9.4	36
31-2	14 - 39	599	8.1	35
31-3	14 - 38	598	8.3	36
31-4	14 - 37	622	8.4	35
31-5	14 - 36	539	7.6	37
31-6	15 - 40	295	4.4	33
31-7	15 - 36	267	5.8	33
Fel 20-1	34 - 59	395	5.9	79
Fel 25	49 - 68	350	7.6	56
Fel 28	60 - 82	94	3.6	43
Fel 28-1 ¹	60 - 82	176	5.5	44
Fel 29	74 - 92	259	5.5	47

1 Based on short form chain 2 sequence (C2S)

Human T cell lines from four cat allergic patients (patients 688, 730, 738, and 807) were isolated. Heparinized peripheral blood specimens were obtained from cat allergic patients and the mononuclear cell fraction was purified by Ficoll-Hypaque centrifugation. An aliquot of these

cells (2×10^6) was stimulated in vitro with purified native TRFP (10 $\mu\text{g/ml}$) and grown in the presence of recombinant IL-2 and IL-4. The T cell line was then rested in culture until ready for secondary
05 proliferation assay.

Two $\times 10^4$ cells were cultured in 200 μl medium containing various concentrations of test antigens with 5×10^4 γ -irradiated autologous Epstein-Barr Virus transformed cells as antigen presenting cells.
10 After 3 days of culture, each microwell was pulsed overnight with 1 μCurie tritiated thymidine, and the amount of radioactivity incorporated was measured by liquid scintillation counting. The stimulation index (S.I.) for each antigen was then calculated. The
15 S.I. is defined as the maximal counts/min for each antigenic stimulation divided by the medium control counts/min.

Two $\times 10^6$ rested T cells were cultured in 1 ml of medium containing 20 $\mu\text{g/ml}$ of test antigen with 2×10^6 γ -irradiated autologous Epstein-Barr Virus transformed cells as antigen presenting cells.
20 Identical controls received no antigen. After a 20 hour incubation, cells were separated from medium by centrifugation. The IL-2 in the medium was measured
25 by proliferation of the IL-2 dependent T cell line, CTLL-3. Cultures of 1×10^4 CTLL-3 were exposed to three dilutions (25%, 5%, 1%) of sample supernatants, incubated for 20 hours, and harvested after 4 hour pulse with 1 μCurie tritiated thymidine. The IL-2
30 bioassay is sensitive to 2 pg/ml IL-2. IL-4 was measured by an ELISA purchased from R&D Systems, Minneapolis, MN. The IL-4 ELISA is sensitive to 16 pg/ml IL-4. The stimulation indices for IL-2 and IL-4 production were calculated by dividing the

amount of IL-2 or IL-4 contained in the medium from stimulated T cells divided by the amount of IL-2 or IL04 released by non-stimulated T cells.

05 The results of these experiments shown in
Figures 15-20. The results show that T-cells from
cat allergic individuals respond to various peptides
derived from the TRFP molecule. The stimulation
index (S.I.) profile for T cell proliferation is
similar to both the IL-2 production profile, and the
10 IL-4 production profile for each of the patients
(i.e. the same peptides shown to induce T cell
proliferation were found to induce IL-2 and IL-4
production.) These results show that T-cell
proliferation, IL-2 and IL-4 production are suitable
15 methods for defining peptides which have T cell
stimulating activity.

Futhermore, none of the epitopes stimulate
IL-4 to the exclusion of IL-2. There do not appear
to be peptides comprising epitopes which stimulate
20 only IL-4, which is the cytokine responsible for IgE
synthesis and subsequent allergic symptoms. However,
one peptide, Fel 4, induces more IL-4 than IL-2 in
this panel of four patients. The data shown in
Figures 15-20 also show that interleukin production
25 favors IL-4 as opposed to IL-2 when T cell lines from
cat allergic individuals were cultured by TRFP.

EQUIVALENTS

Those skilled in the art will recognize or
be able to ascertain using no more than routine
30 experimentation, numerous equivalents to the specific
embodiments described herein. Such equivalents are
considered to be within the scope of this invention
and are encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Gefter, Malcolm L.
Garman, Richard D.
Greenstein, Julia L.
Kuo, Mei-chang
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(ii) TITLE OF INVENTION: PEPTIDES USEFUL FOR TOLERIZATION

(iii) NUMBER OF SEQUENCES: 14

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII TEXT

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/006,116
(B) FILING DATE: 15-JAN-1993

(A) APPLICATION NUMBER: US 07/884,718
(B) FILING DATE: 15-MAY-1992

(A) APPLICATION NUMBER: 07/857,311
(B) FILING DATE: 25-MAR-1992

(viii) ATTORNEY/AGENT INFORMATION:

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- (B) REGISTRATION NUMBER: 36,207
- (C) REFERENCE/DOCKET NUMBER: IPC-031

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (617) 227-7400

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 422 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 8..286

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 74..286

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCATC	ATG	AAG	GGG	GCT	CGT	GTT	CTC	GTG	CTT	CTC	TGG	GCT	GCC	TTG	49	
	Met	Lys	Gly	Ala	Arg	Val	Leu	Val	Leu	Leu	Trp	Ala	Ala	Leu		
	-22		-20					-15					-10			
CTC	TTG	ATC	TGG	GGT	GGA	AAT	TGT	GAA	ATT	TGC	CCA	GCC	GTG	AAG	AGG	97
Leu	Leu	Ile	Trp	Gly	Gly	Asn	Cys	Glu	Ile	Cys	Pro	Ala	Val	Lys	Arg	
		-5					1					5				
GAT	GTT	GAC	CTA	TTC	CTG	ACG	GGA	ACC	CCC	GAC	GAA	TAT	GTT	GAG	CAA	145
Asp	Val	Asp	Leu	Phe	Leu	Thr	Gly	Thr	Pro	Asp	Glu	Tyr	Val	Glu	Gln	
	10					15				20						
GTG	GCA	CAA	TAC	AAA	GCA	CTA	CCT	GTA	GTA	TTG	GAA	AAT	GCC	AGA	ATA	193
Val	Ala	Gln	Tyr	Lys	Ala	Leu	Pro	Val	Val	Leu	Glu	Asn	Ala	Arg	Ile	
	25				30					35					40	
CTG	AAG	AAC	TGC	GTT	GAT	GCA	AAA	ATG	ACA	GAA	GAG	GAT	AAG	GAG	AAT	241
Leu	Lys	Asn	Cys	Val	Asp	Ala	Lys	Met	Thr	Glu	Glu	Asp	Lys	Glu	Asn	
			45					50						55		

GCT CTC AGC TTG CTG GAC AAA ATA TAC ACA AGT CCT CTG TGT TAAAGGAGCC	293
Ala Leu Ser Leu Leu Asp Lys Ile Tyr Thr Ser Pro Leu Cys	
60 65 70	
ATCACTGCCA GGAGCCCTAA GGAAGCCACT GAACTGATCA CTAAGTAGTC TCAGCAGCCT	353
GCCATGTCCA GGTGTCTTAC TAGAGGATTC CAGCAATAAA AGCCTTGCAA TTCAAACAAA	413
AAAAAAAAA	422

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 92 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Lys Gly Ala Arg Val Leu Val Leu Leu Trp Ala Ala Leu Leu Leu	
-22 -20 -15 -10	
Ile Trp Gly Gly Asn Cys Glu Ile Cys Pro Ala Val Lys Arg Asp Val	
-5 1 5 10	
Asp Leu Phe Leu Thr Gly Thr Pro Asp Glu Tyr Val Glu Gln Val Ala	
15 20 25	
Gln Tyr Lys Ala Leu Pro Val Val Leu Glu Asn Ala Arg Ile Leu Lys	
30 35 40	
Asn Cys Val Asp Ala Lys Met Thr Glu Glu Asp Lys Glu Asn Ala Leu	
45 50 55	
Ser Leu Leu Asp Lys Ile Tyr Thr Ser Pro Leu Cys	
60 65 70	

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 428 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

-70-

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 80..292

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 26..292

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGCCTGGCGG TGCTCCTGGA AAAGG ATG TTA GAC GCA GCC CTC CCA CCC TGC	52
Met Leu Asp Ala Ala Leu Pro Pro Cys	
-18 -15 -10	
CCT ACT GTT GCG GCC ACA GCA GAT TGT GAA ATT TGC CCA GCC GTG AAG	100
Pro Thr Val Ala Ala Thr Ala Asp Cys Glu Ile Cys Pro Ala Val Lys	
-5 1 5	
AGG GAT GTT GAC CTA TTC CTG ACG GGA ACC CCC GAC GAA TAT GTT GAG	148
Arg Asp Val Asp Leu Phe Leu Thr Gly Thr Pro Asp Glu Tyr Val Glu	
10 15 20	
CAA GTG GCA CAA TAC AAA GCA CTA CCT GTA GTA TTG GAA AAT GCC AGA	196
Gln Val Ala Gln Tyr Lys Ala Leu Pro Val Val Leu Glu Asn Ala Arg	
25 30 35	
ATA CTG AAG AAC TGC GTT GAT GCA AAA ATG ACA GAA GAG GAT AAG GAG	244
Ile Leu Lys Asn Cys Val Asp Ala Lys Met Thr Glu Glu Asp Lys Glu	
40 45 50 55	
AAT GCT CTC AGC TTG CTG GAC AAA ATA TAC ACA AGT CCT CTG TGT TAA	292
Asn Ala Leu Ser Leu Leu Asp Lys Ile Tyr Thr Ser Pro Leu Cys	
60 65 70	
AGGAGCCATC ACTGCCAGGA GCCCTAAGGA AGCCACTGAA CTGATCACTA AGTAGTCTCA	352
GCAGCCTGCC ATGTCCAGGT GTCTTACTAG AGGATTCCAG CAATAAAAGC CTTGCAATTC	412
AAACAAAAAA AAAAAA	428

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 88 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

-71-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Leu Asp Ala Ala Leu Pro Pro Cys Pro Thr Val Ala Ala Thr Ala
-18          -15          -10          -5

Asp Cys Glu Ile Cys Pro Ala Val Lys Arg Asp Val Asp Leu Phe Leu
      1              5              10

Thr Gly Thr Pro Asp Glu Tyr Val Glu Gln Val Ala Gln Tyr Lys Ala
15          20          25          30

Leu Pro Val Val Leu Glu Asn Ala Arg Ile Leu Lys Asn Cys Val Asp
          35          40          45

Ala Lys Met Thr Glu Glu Asp Lys Glu Asn Ala Leu Ser Leu Leu Asp
          50          55          60

Lys Ile Tyr Thr Ser Pro Leu Cys
      65          70

```

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 485 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:
- (A) NAME/KEY: CDS
 - (B) LOCATION: 8..337

- (ix) FEATURE:
- (A) NAME/KEY: mat_peptide
 - (B) LOCATION: 59..337

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

TGACACG ATG AGG GGG GCA CTG CTT GTG CTG GCA TTG CTG GTG ACC CAA
Met Arg Gly Ala Leu Leu Val Leu Ala Leu Leu Val Thr Gln
-17    -15          -10          -5

GCG CTG GGC GTC AAG ATG GCG GAA ACT TGC CCC ATT TTT TAT GAC GTC
Ala Leu Gly Val Lys Met Ala Glu Thr Cys Pro Ile Phe Tyr Asp Val
      1              5              10

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49

97

TTT TTT GCG GTG GCC AAT GGA AAT GAA TTA CTG TTG GAC TTG TCC CTC	145
Phe Phe Ala Val Ala Asn Gly Asn Glu Leu Leu Leu Asp Leu Ser Leu	
15 20 25	
ACA AAA GTC AAT GCT ACT GAA CCA GAG AGA ACA GCC ATG AAA AAA ATC	193
Thr Lys Val Asn Ala Thr Glu Pro Glu Arg Thr Ala Met Lys Lys Ile	
30 35 40 45	
CAG GAT TGC TAC GTG GAG AAC GGA CTC ATA TCC AGG GTC TTG GAT GGA	241
Gln Asp Cys Tyr Val Glu Asn Gly Leu Ile Ser Arg Val Leu Asp Gly	
50 55 60	
CTA GTC ATG ACA ACC ATC AGC TCC AGC AAA GAT TGC ATG GGT GAA GCA	289
Leu Val Met Thr Thr Ile Ser Ser Ser Lys Asp Cys Met Gly Glu Ala	
65 70 75	
GTT CAG AAC ACC GTA GAA GAT CTC AAG CTG AAC ACT TTG GGG AGA TGA	337
Val Gln Asn Thr Val Glu Asp Leu Lys Leu Asn Thr Leu Gly Arg	
80 85 90	
ATTTTGCCAC TGATGCCCCCT TCTGAGCCCC ATCCTCCTGC CCTGTTCTTT ACACCTAAAG	397
CTGGAATCCA GACACCTGTC CTCACCTAAT TCACTCTCAA TCAGGCTGAC TAGAATAAAA	457
TAACTGCATC TTAAAAAAA AAAAAAAA	485

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 109 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Arg Gly Ala Leu Leu Val Leu Ala Leu Leu Val Thr Gln Ala Leu	
-17 -15 -10 -5	
Gly Val Lys Met Ala Glu Thr Cys Pro Ile Phe Tyr Asp Val Phe Phe	
1 5 10 15	
Ala Val Ala Asn Gly Asn Glu Leu Leu Leu Asp Leu Ser Leu Thr Lys	
20 25 30	
Val Asn Ala Thr Glu Pro Glu Arg Thr Ala Met Lys Lys Ile Gln Asp	
35 40 45	

Cys Tyr Val Glu Asn Gly Leu Ile Ser Arg Val Leu Asp Gly Leu Val
 50 55 60
 Met Thr Thr Ile Ser Ser Ser Lys Asp Cys Met Gly Glu Ala Val Gln
 65 70 75
 Asn Thr Val Glu Asp Leu Lys Leu Asn Thr Leu Gly Arg
 80 85 90

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys Arg Asp Val Asp Leu Phe Leu Thr Gly Thr Pro Asp Glu Tyr Val
 1 5 10 15
 Glu Gln Val Ala Gln Tyr Lys Ala Leu Pro Val
 20 25

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Lys Ala Leu Pro Val Val Leu Glu Asn Ala Arg Ile Leu Lys Asn Cys
 1 5 10 15
 Val Asp Ala Lys Met Thr Glu Glu Asp Lys Glu
 20 25

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Phe	Phe	Ala	Val	Ala	Asn	Gly	Asn	Glu	Leu	Leu	Leu	Asp	Leu	Ser	Leu
1				5				10						15	
Thr	Lys	Val	Asn	Ala	Thr	Glu	Pro	Glu	Arg						
			20					25							

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Glu	Glu	Asp	Lys	Glu	Asn	Ala	Leu	Ser	Leu	Leu	Asp	Lys	Ile	Tyr	Thr
1				5				10						15	
Ser	Pro	Leu													

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Gly Glu Ala Val Gln Asn Thr Val Glu Asp Leu Lys Leu Asn Thr
1 5 10 15

Leu Gly Arg

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Glu Glu Asp Lys Glu Asn Ala Leu Ser Leu Leu Asp Lys Ile Tyr Thr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Asn Ala Leu Ser Leu Leu Asp Lys Ile Tyr Thr Ser Pro Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Thr	Glu	Glu	Asp	Lys	Glu	Asn	Ala	Leu	Ser	Leu	Leu	Asp	Lys	Ile	Tyr
1				5					10					15	

Thr	Ser	Pro	Leu
			20

Claims

1. A composition comprising at least one peptide derived from a human T cell reactive feline protein, said peptide selected from the group consisting of peptide X (SEQ ID NO: 7), peptide Y (SEQ ID NO: 8), peptide Z (SEQ ID NO: 9), peptide A (SEQ ID NO: 10), peptide B (SEQ ID NO: 11), peptide C (SEQ ID NO: 12), peptide D (SEQ ID NO: 13), and peptide E (SEQ ID NO: 14), each as shown in Figure 3.
2. The composition of claim 1 wherein the at least one peptide is selected from the group consisting of peptide X (SEQ ID NO: 7), peptide Y (SEQ ID NO: 8), peptide Z (SEQ ID NO: 9), peptide A (SEQ ID NO: 10), peptide B (SEQ ID NO: 11) and peptide E (SEQ ID NO: 14).
3. A composition of claim 2, wherein the composition comprises peptide X (SEQ ID NO: 7).
4. A composition of claim 2, wherein the composition comprises peptide Y (SEQ ID NO: 8).
5. A composition of claim 1, wherein said composition comprises at least two peptides selected from the group consisting of peptide X (SEQ ID NO: 7), peptide Y (SEQ ID NO: 8), peptide Z (SEQ ID NO: 9), peptide A (SEQ ID NO: 10), peptide B (SEQ ID NO: 11) and peptide E (SEQ ID NO: 14).

6. A composition of claim 5, wherein the composition comprises peptide X (SEQ ID NO: 7) and peptide Y (SEQ ID NO: 8).
7. A therapeutic composition comprising a pharmaceutically acceptable carrier or diluent and at least one peptide derived from a human T cell reactive feline protein, said peptide selected from the group consisting of peptide X, peptide Y (SEQ ID NO: 8), peptide Z (SEQ ID NO: 9), peptide A (SEQ ID NO: 10), peptide B (SEQ ID NO: 11), peptide C (SEQ ID NO: 12), peptide D (SEQ ID NO: 13) and peptide E (SEQ ID NO: 14), each as shown in Figure 3.
8. A therapeutic composition of claim 7 wherein said at least one peptide is selected from the group consisting of peptide X (SEQ ID NO: 7), peptide Y (SEQ ID NO: 8), peptide Z (SEQ ID NO: 9), peptide A (SEQ ID NO: 10), peptide B (SEQ ID NO: 11) and peptide E (SEQ ID NO: 14).
9. A therapeutic composition of claim 8 wherein the composition comprises peptide X (SEQ ID NO: 7).
10. A therapeutic composition of claim 8 wherein the composition comprises peptide Y (SEQ ID NO: 8).
11. A therapeutic composition of claim 8 wherein said composition comprises at least two peptides selected from the group consisting of peptide X (SEQ ID NO: 7), peptide Y (SEQ ID NO: 8), peptide Z (SEQ ID NO: 9), peptide A (SEQ ID NO: 10), peptide B (SEQ ID NO: 11) and peptide E (SEQ ID NO: 14).

12. A therapeutic composition of claim 11 wherein the composition comprises peptide X (SEQ ID NO: 7) and peptide Y (SEQ ID NO: 8).
13. A method of treating sensitivity to Felis domesticus in an individual comprising administering to the individual a therapeutically effective amount of the composition of claim 7.
14. A method of claim 13 wherein the therapeutic composition is administered subcutaneously.
15. A method of claim 13 wherein the therapeutic composition is administered in soluble form.
16. A method of treating sensitivity to Felis domesticus in an individual comprising administering to the individual a therapeutically effective amount of the composition of claim 8.
17. A method of claim 16 wherein the therapeutic composition is administered subcutaneously.
18. A method of claim 16 wherein the therapeutic composition is administered in soluble form.
19. A method of treating sensitivity to Felis domesticus in an individual comprising administering to the individual a therapeutically effective amount of the composition of claim 12.

20. A method of claim 19 wherein the therapeutic composition is administered subcutaneously.
21. A method of claim 19 wherein the therapeutic composition is administered in soluble form.
22. A method of treating sensitivity to Felis domesticus in an individual comprising administering to the individual a therapeutically effective amount of at least two therapeutic compositions, each composition comprising at least one peptide selected from the group consisting of peptide X (SEQ ID NO: 7), peptide Y (SEQ ID NO: 8), peptide Z (SEQ ID NO: 9), peptide A (SEQ ID NO: 10), peptide B (SEQ ID NO: 11) and peptide E (SEQ ID NO: 14), each peptide shown in Figure 3, said compositions each comprising a pharmaceutically acceptable carrier or diluent.
23. A method of claim 22, wherein one of the therapeutic compositions comprises peptide X (SEQ ID NO: 7) and one of the therapeutic compositions comprises peptide Y (SEQ ID NO: 8).
24. A method off claim 22 wherein each composition is administered in soluble form.

25. A therapeutic composition useful in treating a disease which involves an immune response to a protein antigen, said therapeutic composition comprising at least one peptide which comprises a sufficient percentage of the T cell epitopes of said protein antigen such that in a substantial percentage of a population of individuals sensitive to the protein antigen, the response of such individuals to the protein antigen is substantially diminished, with the proviso that the at least one peptide does not comprise the entire protein antigen.
26. The therapeutic composition of claim 25 wherein the response of the T cells of such individuals to said protein antigen is substantially diminished.
27. The therapeutic composition of claim 25 wherein the protein antigen is an allergen.
28. The therapeutic composition of claim 25 wherein said therapeutic composition comprises at least two compositions.
29. A method of treating a disease which involves an immune response to a protein antigen comprising administering subcutaneously to a mammal a therapeutic composition comprising a peptide derived from the protein antigen and a pharmaceutically acceptable carrier, in an amount effective to tolerize T cells of the mammal to the protein antigen, wherein the peptide comprises at least one T cell epitope of the protein antigen.

30. A method of claim 29 wherein the mammal is a human.
31. A method of claim 30 wherein the protein antigen is an allergen.
32. A method of claim 31 wherein the allergen is from a genus selected from the group consisting of: the genus Dermatophagoides; the genus Felis; the genus Ambrosia; the genus Lolium; the genus Cryptomeria; the genus Alternaria; the genus Alder; the genus Betula; the genus Quercus; the genus Olea; the genus Artemisia; the genus Plantago; the genus Parietaria; the genus Canis; the genus Blattella; the genus Apis; and the genus Periplaneta.
33. A method of claim 32 wherein the allergen is selected from the group consisting of:
Der p I; Der f I; Der f II; Amb a I; Amb a II;
Lol p I; Lol p IX; Cry i I; and Cry i II.
34. A method of claim 31 wherein the peptide has minimal immunoglobulin E stimulating activity.
35. A method of claim 31 wherein the peptide binds immunoglobulin E to a substantially lesser extent than protein allergen from which the peptide is derived binds said immunoglobulin E.

36. A method of claim 31 wherein the peptide does not bind immunoglobulin E specific for the protein allergen from which it is derived binds said immunoglobulin E, or if binding of the peptide to said immunoglobulin E occurs, such binding does not result in release of mediators from mast cells or basophils in a substantial percentage of individuals sensitive to said protein allergen.
37. A method of claim 31 wherein the peptide is selected from the group consisting of peptide X (SEQ ID NO: 7); peptide Y (SEQ NO: 8); peptide Z (SEQ ID NO: 9); peptide A (SEQ ID NO: 10); peptide B (SEQ ID NO: 11); and peptide E (SEQ ID NO: 14), each peptide as shown in Fig. 3.
38. A method of claim 30 wherein the protein antigen is an autoantigen.
39. A method of claim 38 wherein the autoantigen is selected from the group consisting of: insulin; myelin basic protein; rh factor; acetylcholine receptors; thyroid cell receptors; basement membrane proteins; thyroid proteins; PM-1; glutamic acid decarboxylase (64K); and carboxypeptidase H.
40. A method of claim 38 wherein the autoantigen is human myelin basic protein and the peptide consists essentially of amino acid residues 84-106 of human myelin basic protein.

41. A method of claim 38 wherein the autoantigen is human myelin basic protein and the peptide consists essentially of amino acid residues 89-101 of human myelin basic protein.
42. A method of claim 38 wherein the autoantigen is human myelin basic protein and the peptide consists essentially of amino acid residues 140-172 of human myelin basic protein.
43. A method of claim 38 wherein the autoantigen is human myelin basic protein and the peptide consists essentially of amino acid residues 143-168 of human myelin basic protein.
44. A method of claim 30 wherein the composition is administered in soluble form.

58
TGACACGATGAGGGGGCAGCTGCTTGCTGGCAATTGCTGGTACCCCAAGCGCTGGGC
M R G A L L V L A L L V T Q A L G
-17 -15 -10 -5
140
GTCAAGATGGCGGMAACTTGCCCATTTTNTGACGCTCTTTTGGGTGGCCATGGNATGNATTACTGTGGACTTGCCCTCACA
V K H A E T C P I F Y D V F A V A N G N E L L L D L S L T
1 5 10 15 20 25 30
238
AAGTCAATGCTACTGACAGAGAGACGCCATGMAAATCCAGGATGCTACGTGGAGMACGGACTCATATCCAGGGCTTGGAT
K V N A T E P E R T A N K K I Q D C Y V E N G L I S R V L D
35 40 45 50 55 60
328
GGACTAGTCNTGACMACCAACAGCTCCAGCAGCAAGATTGCATGGTGAGCAGTTCAGMACCCGTAGMAGATCTCAGCTGACACTTTG
G L V H T T I S S K D C M G E A V Q N T V E D L K L N T L
65 70 75 80 85 90
418
GGGAGATGMAATTTGCCACTGNTGCCCTTCTGAGCCCCCATCTCTGCGCCCTGTTCTTTACACCTNAGCTGGMATCCAGACACCTGTCC
G R -
485
TCACCTAATTCACCTCMTACAGGCTGACTAGATATAATATACGTCATCTTAAAAA

FIG. 2

Sequence

peptide
name

X	KRDVDLFLTGTPDEYVEQVAQYKALPV
Y	KALPVVLENARILKNCVDAKMTEEDKE
Z	FFAVANGNELLLDLSLTKVNATEPER
A	EEDKENALSLLDKIYTSPL
B	MGEAVQNTVEDLKLNTLGR
C	EEDKENALSLLDKIYT
D	NALSLLDKIYTSPL
E	TEEDKENALSLLDKIYTSPL

FIG. 3

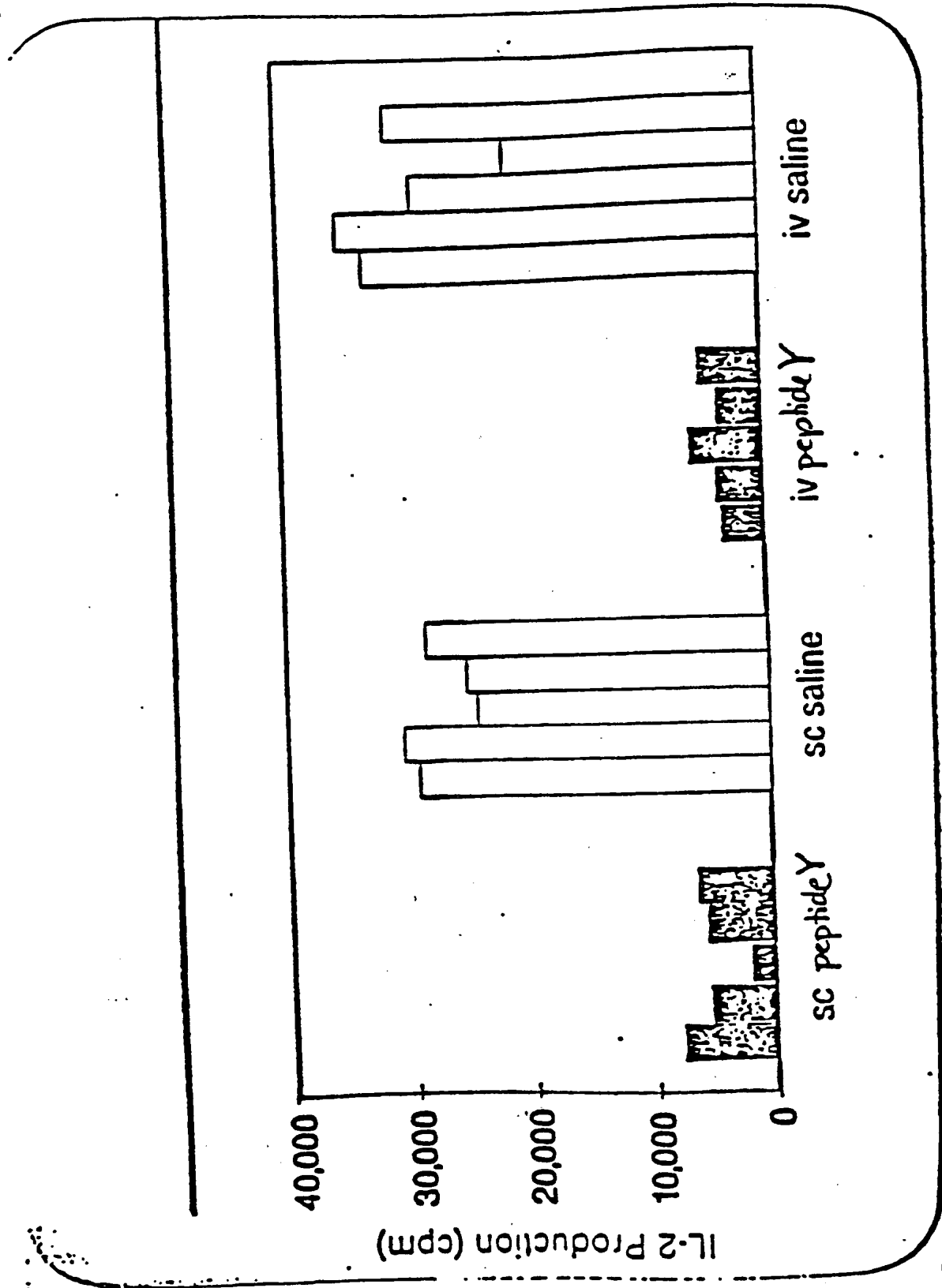


Fig. 4

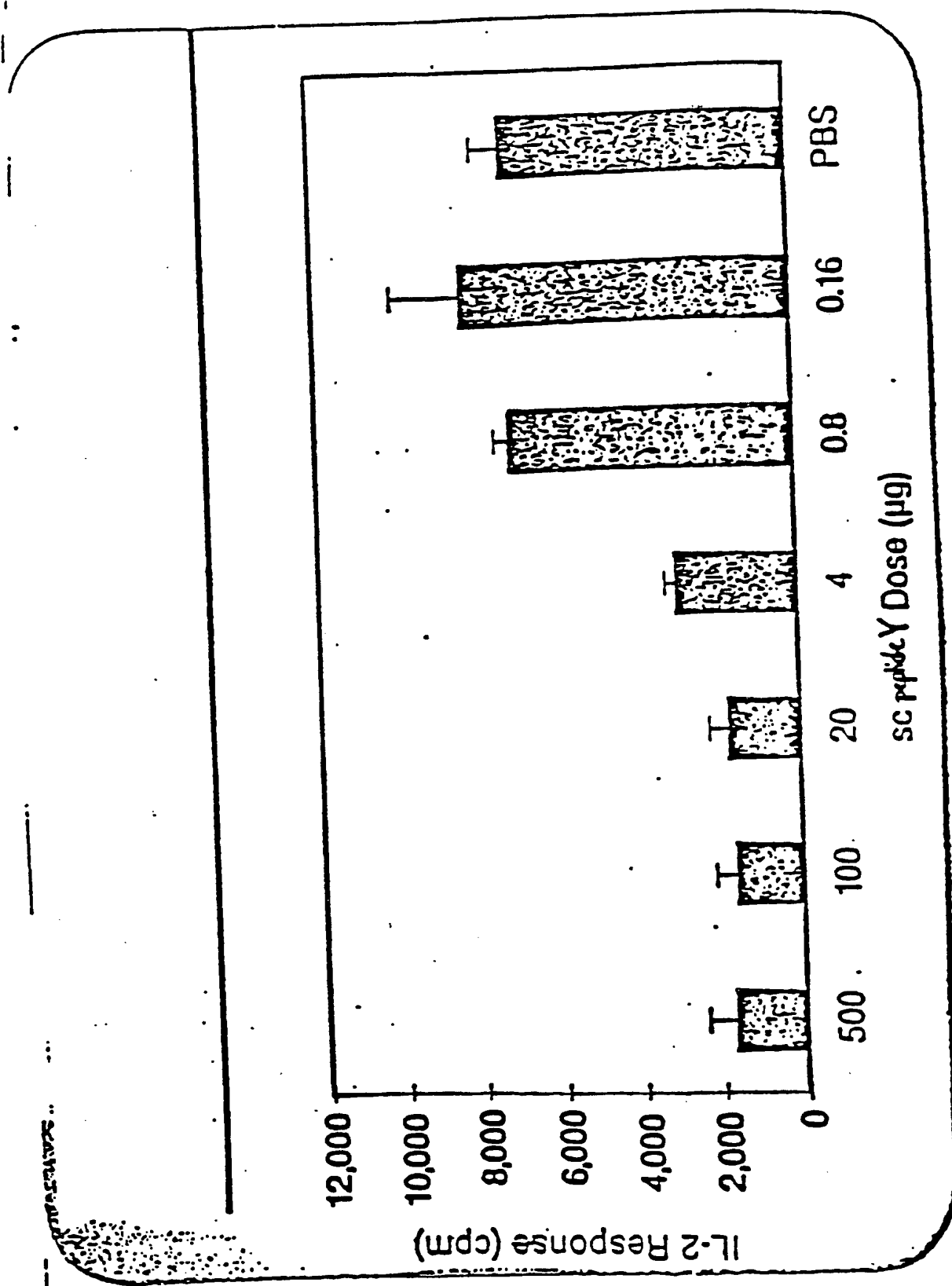


FIG. 5

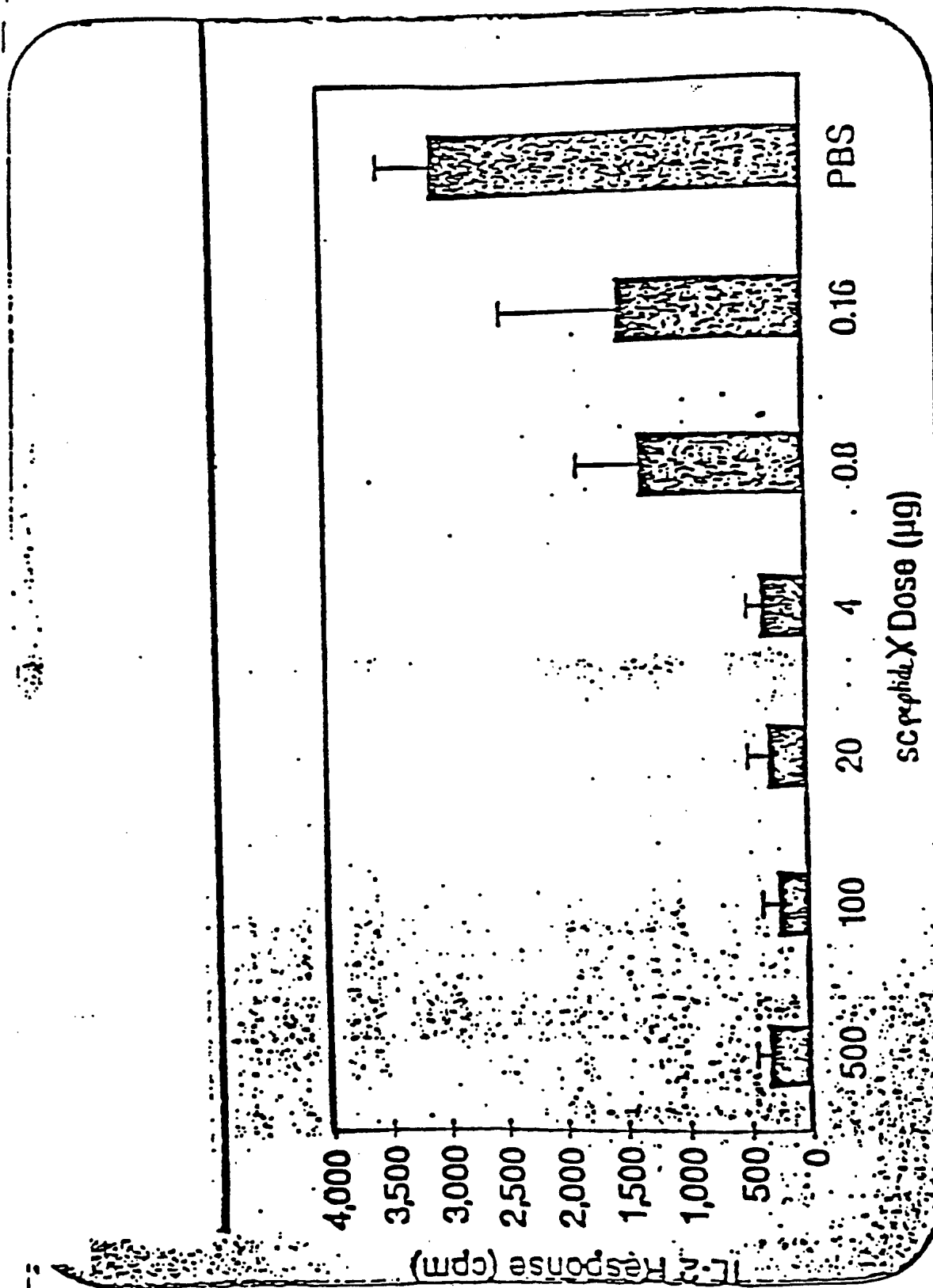


FIG. 6

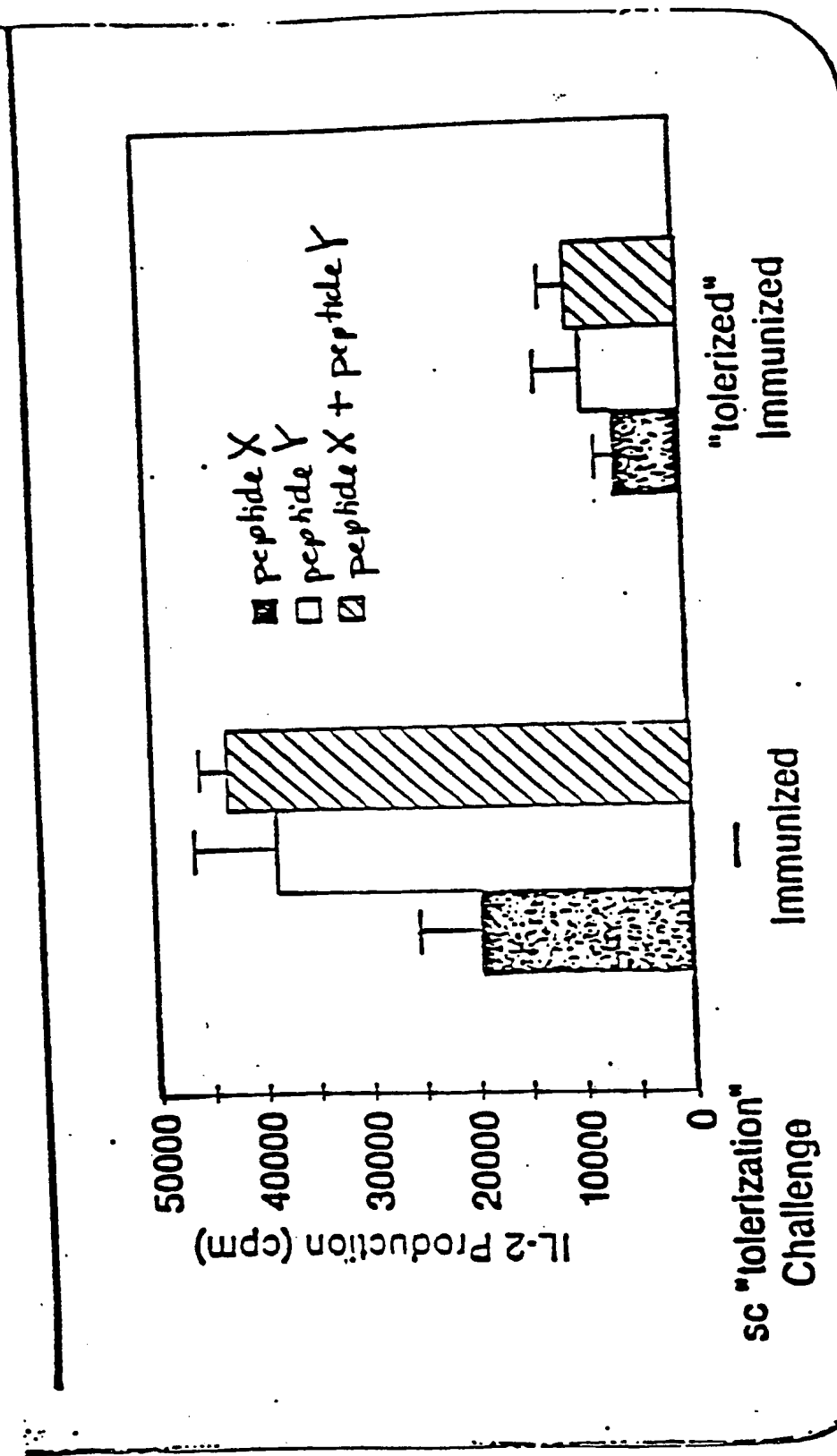


Fig. 7

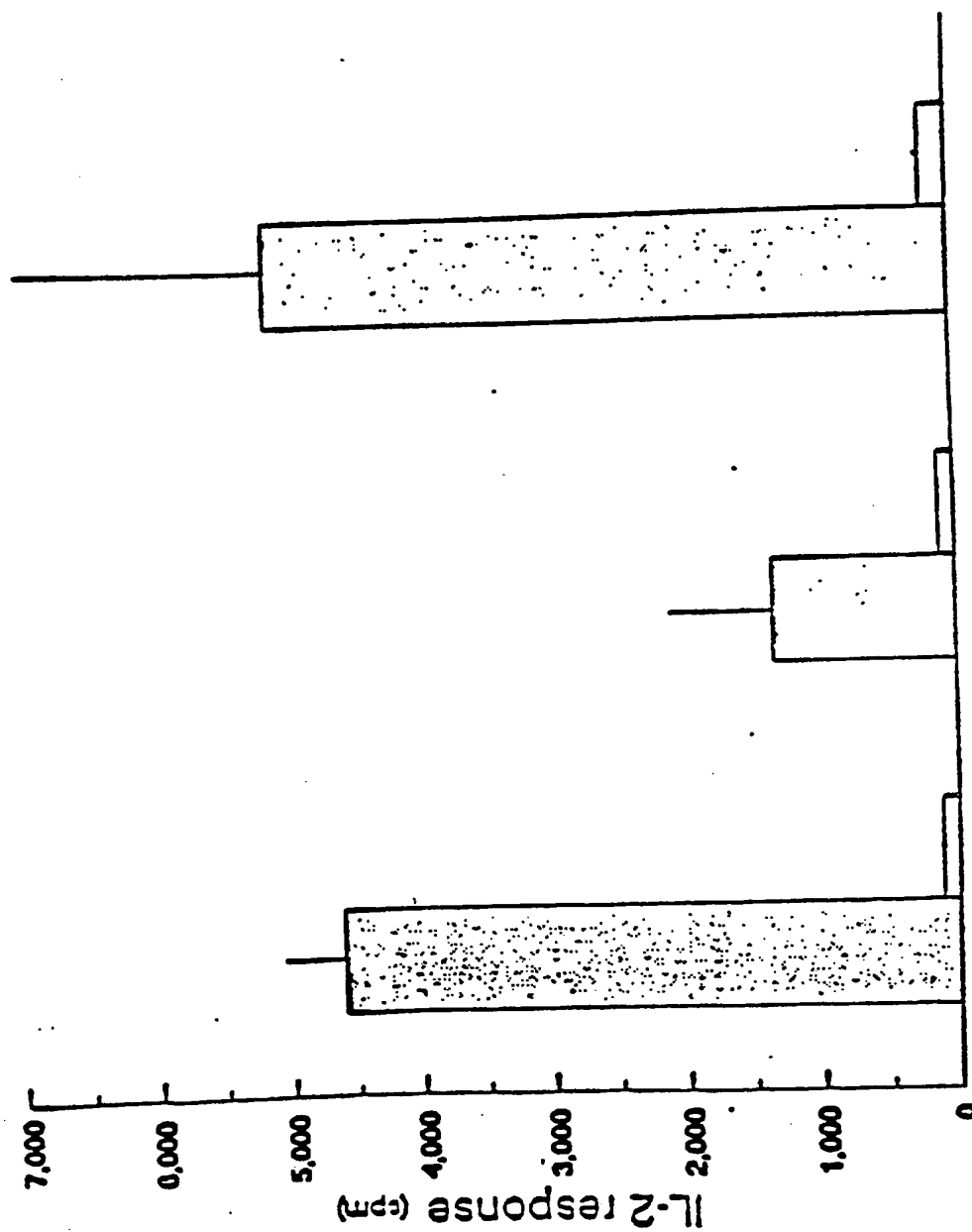


Fig. 8

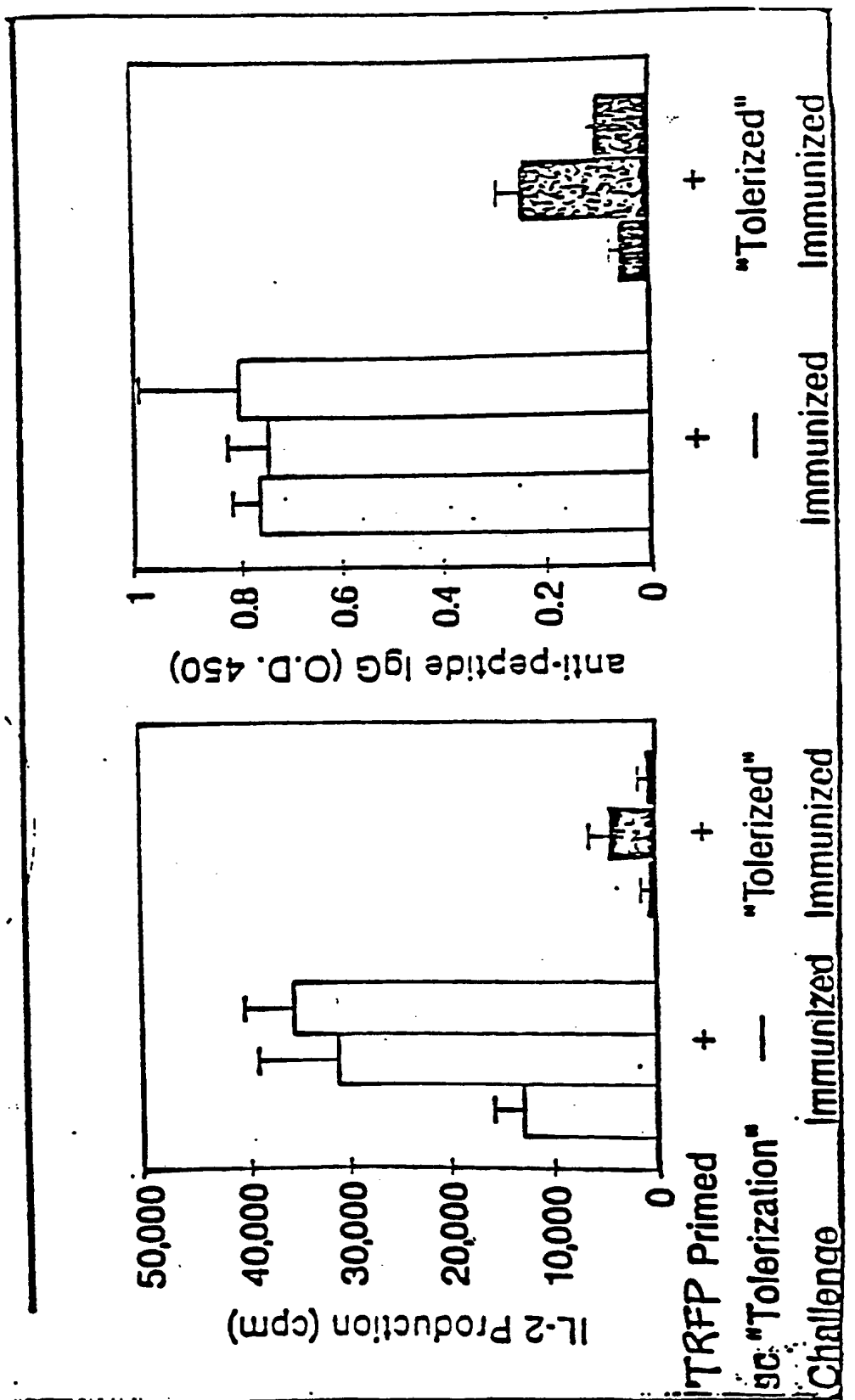
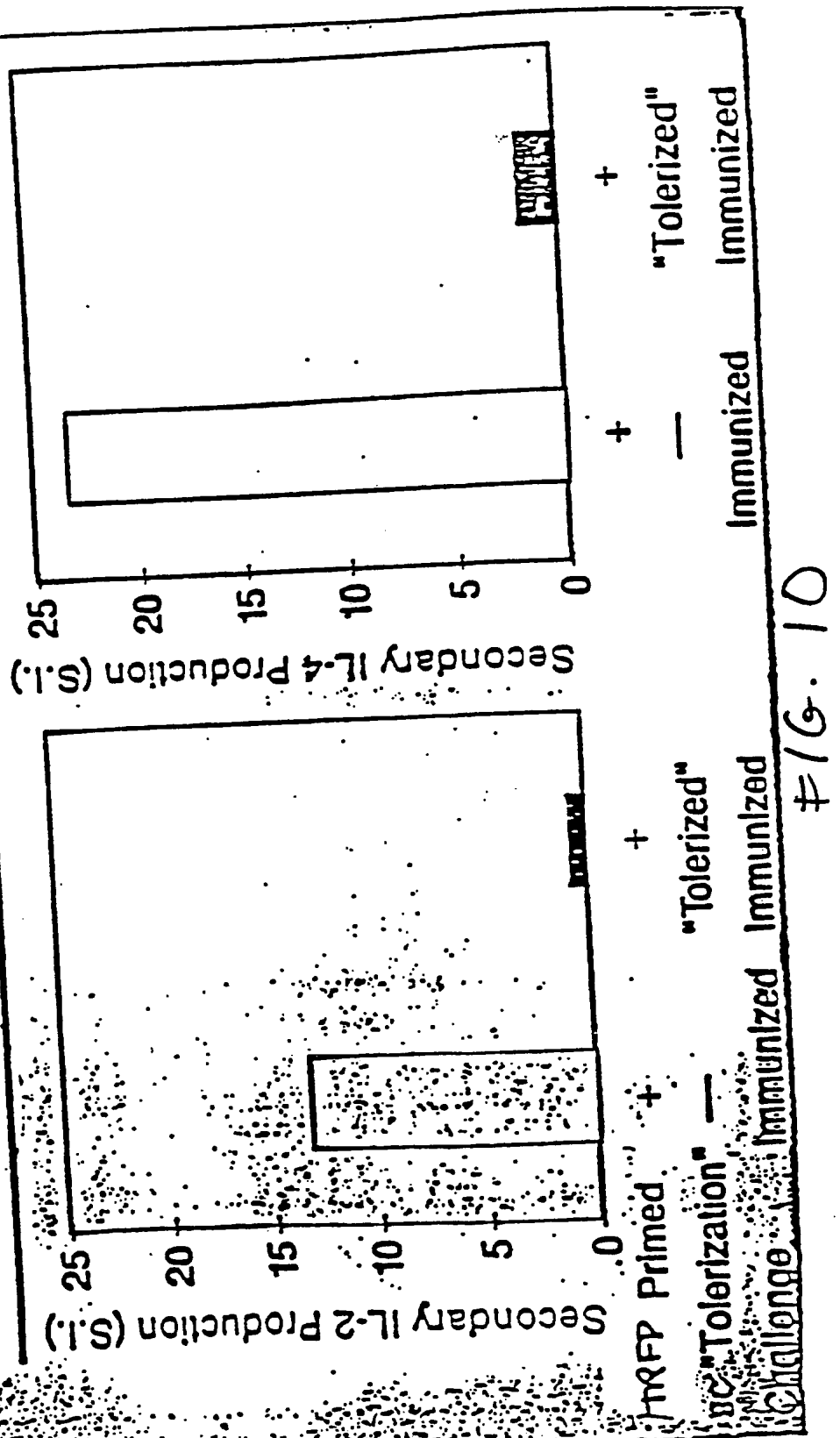


FIG. 9



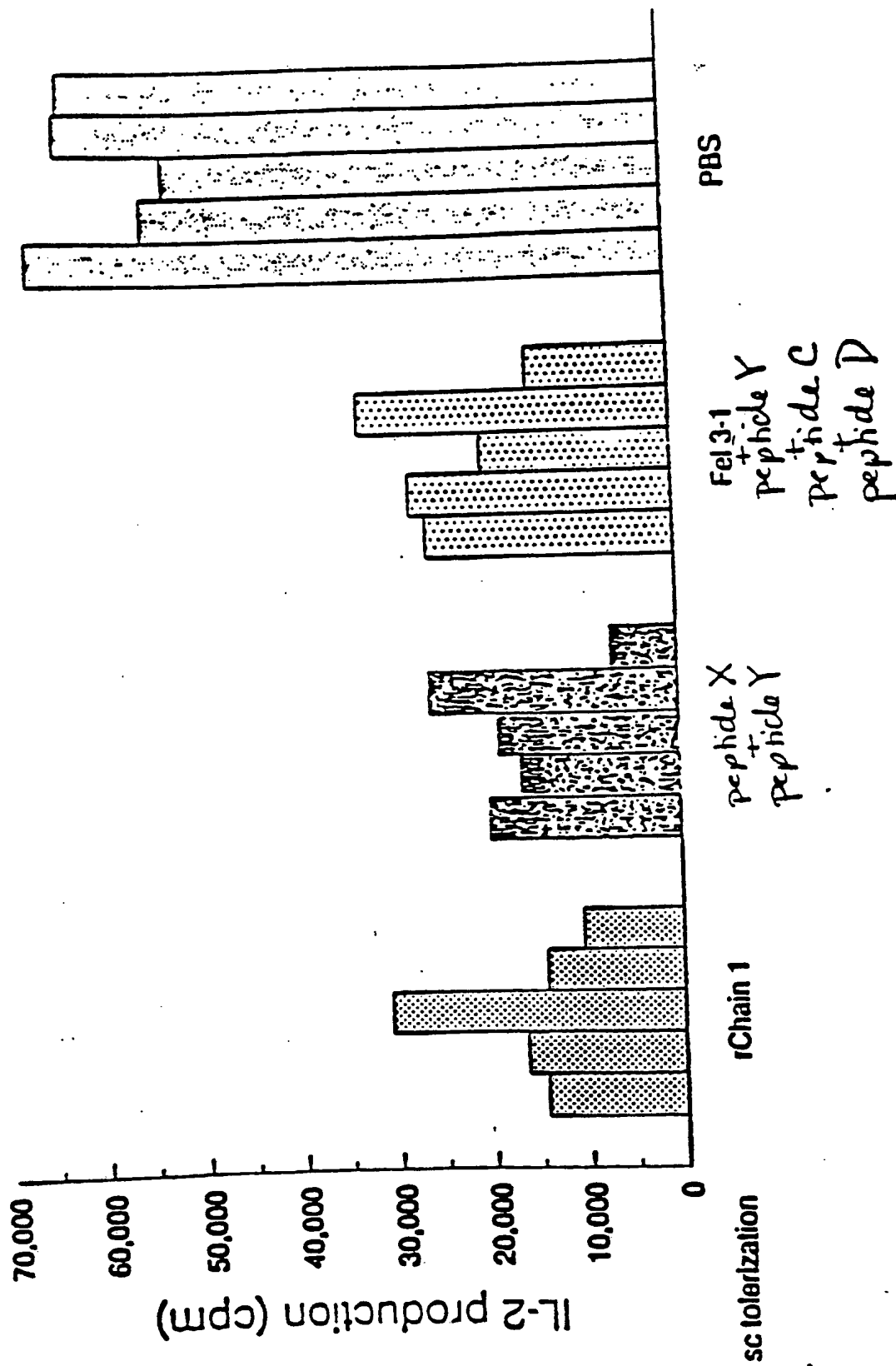


FIG. 11

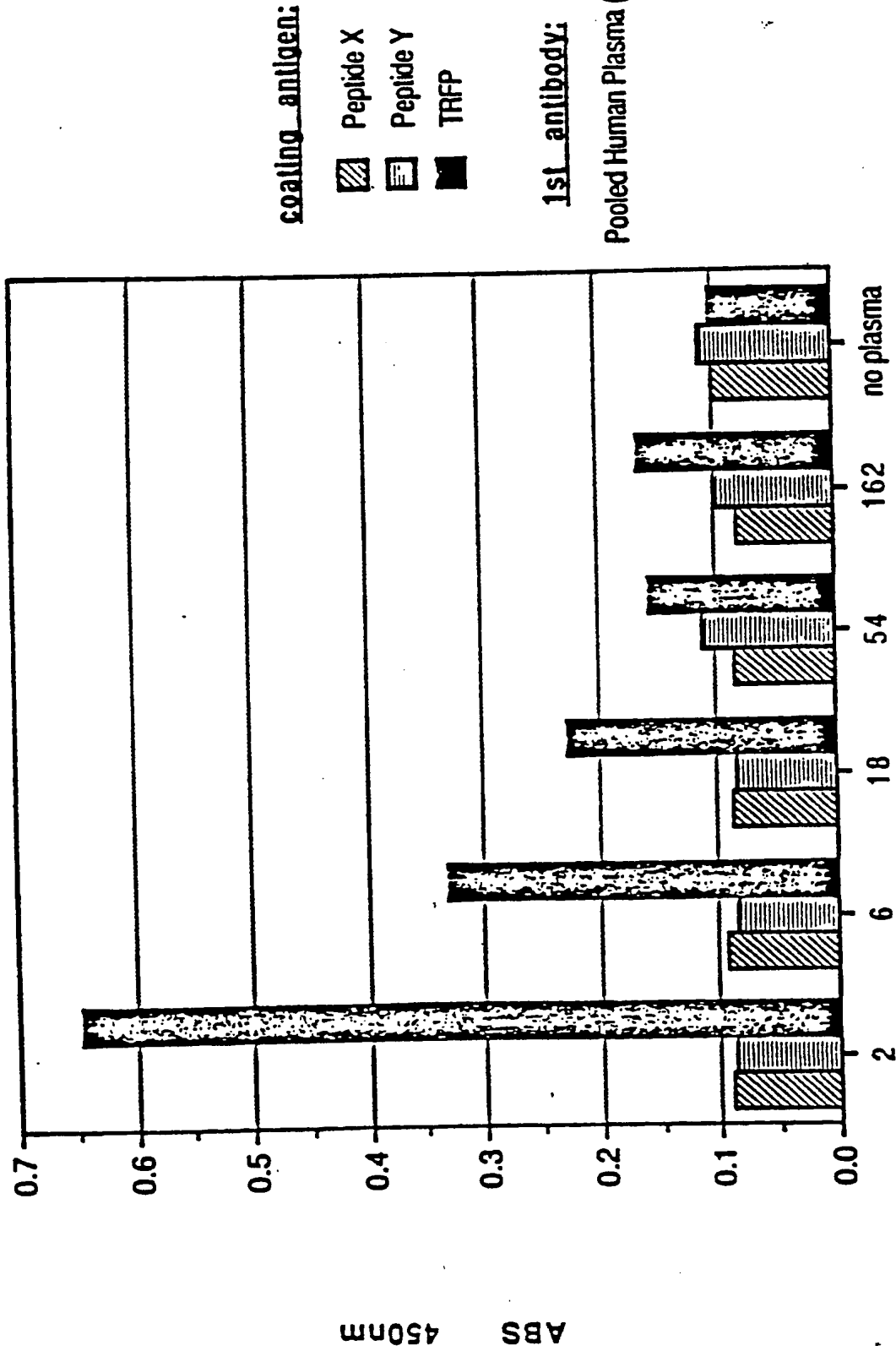


FIG. 12

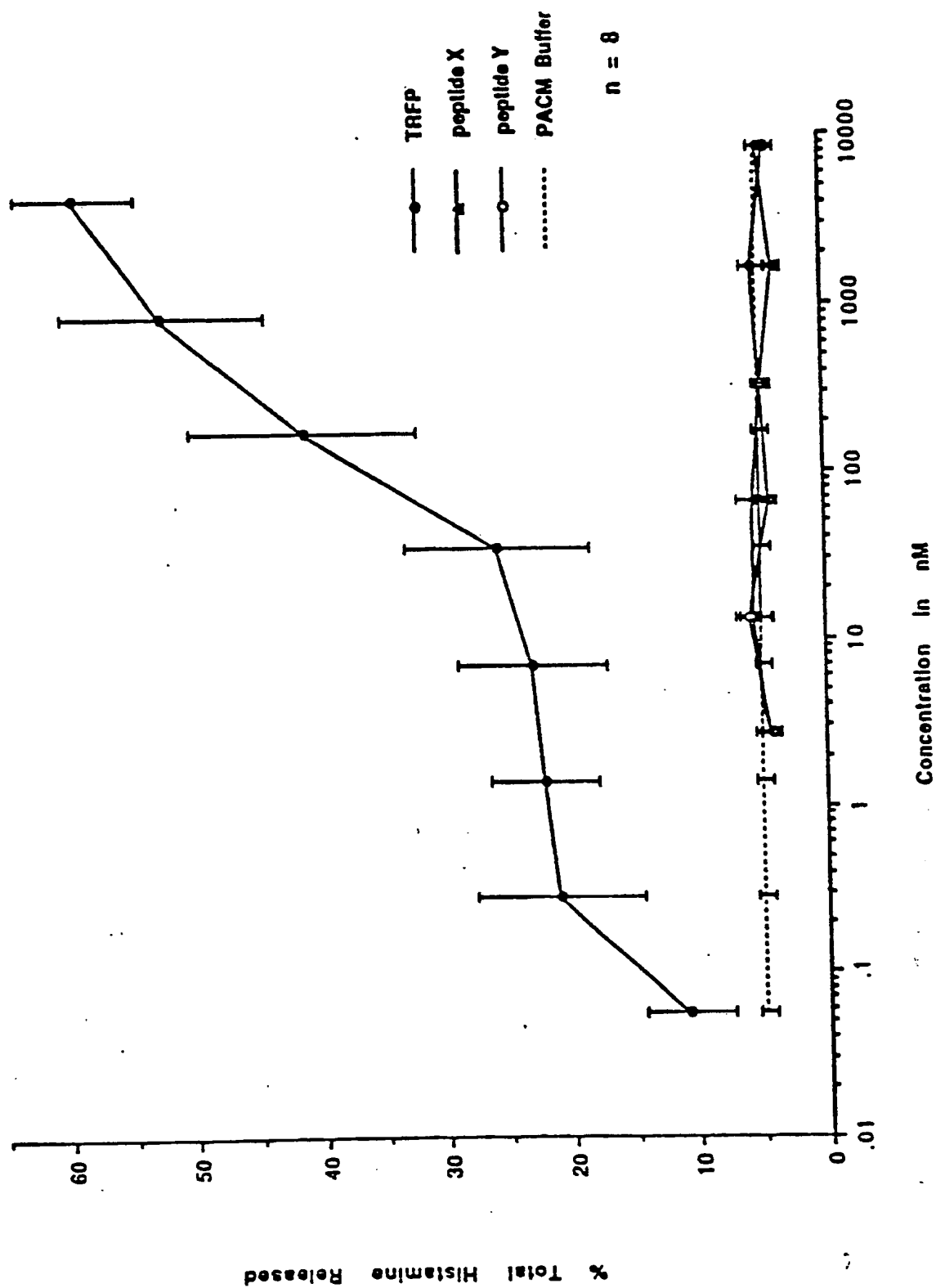


FIG. 13

Fel 32 VKMAETCPIFYDVFFAVA
Fel 33 FYDVFFAVANGNELLLD
Fel 34 NGNELLLDLSLTKVNATE
Fel 35 SLTKVNATEPERTAMKKI
Fel 36 ERTAMKKIQDCYVENGL
Fel 37 QDCYVENGLISRVL DGLV
Fel 38 ISRVL DGLVMTTISSSKDCM
Fel 38-1 ISRVL DGLVMIAINE**DCM
Fel 39 MTTISSSKDCMGEAVQNTTEVELDKLNTLGF
Fel 39.1 MIAINE**DCMGEAVQNTTEVELDKLNTLGF

FIG 14.

Patient 688

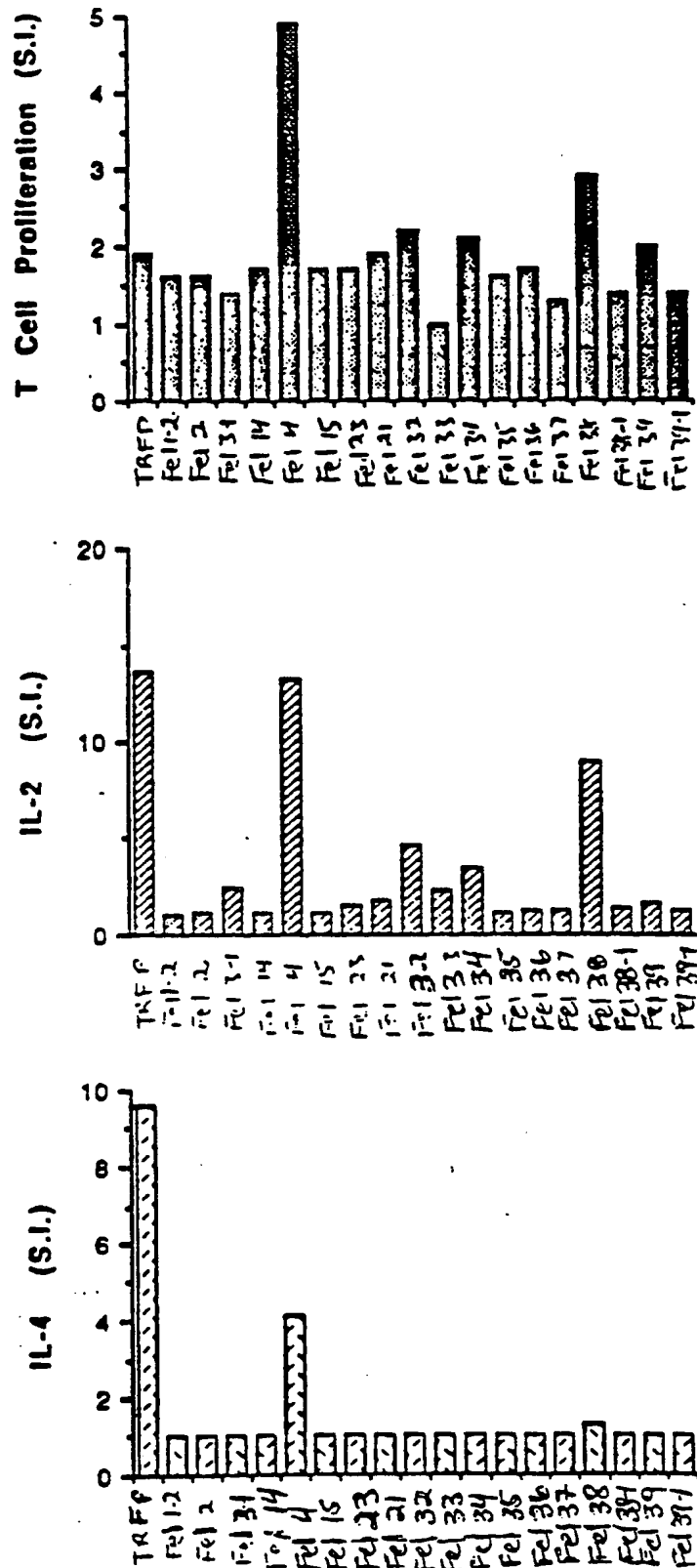


FIG. 15

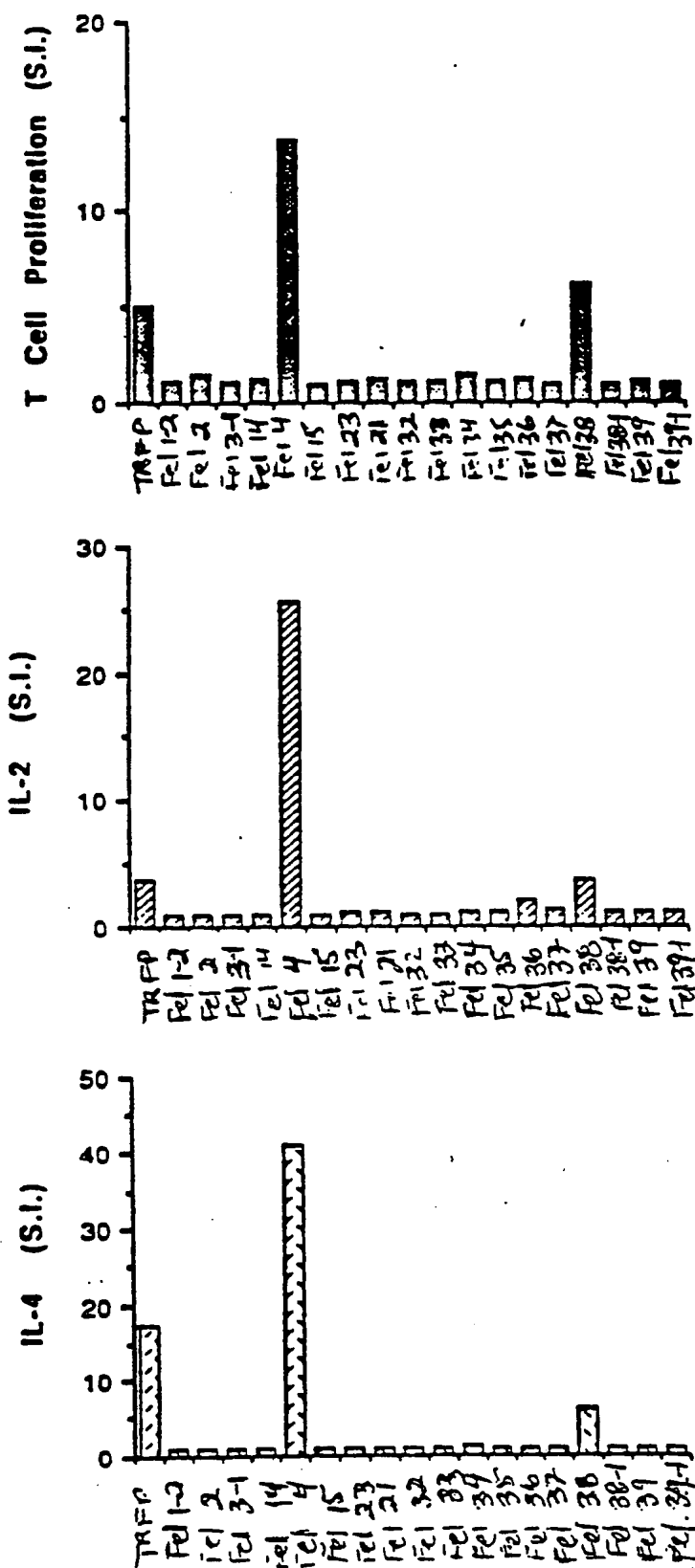


FIG. 16

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Patient 738

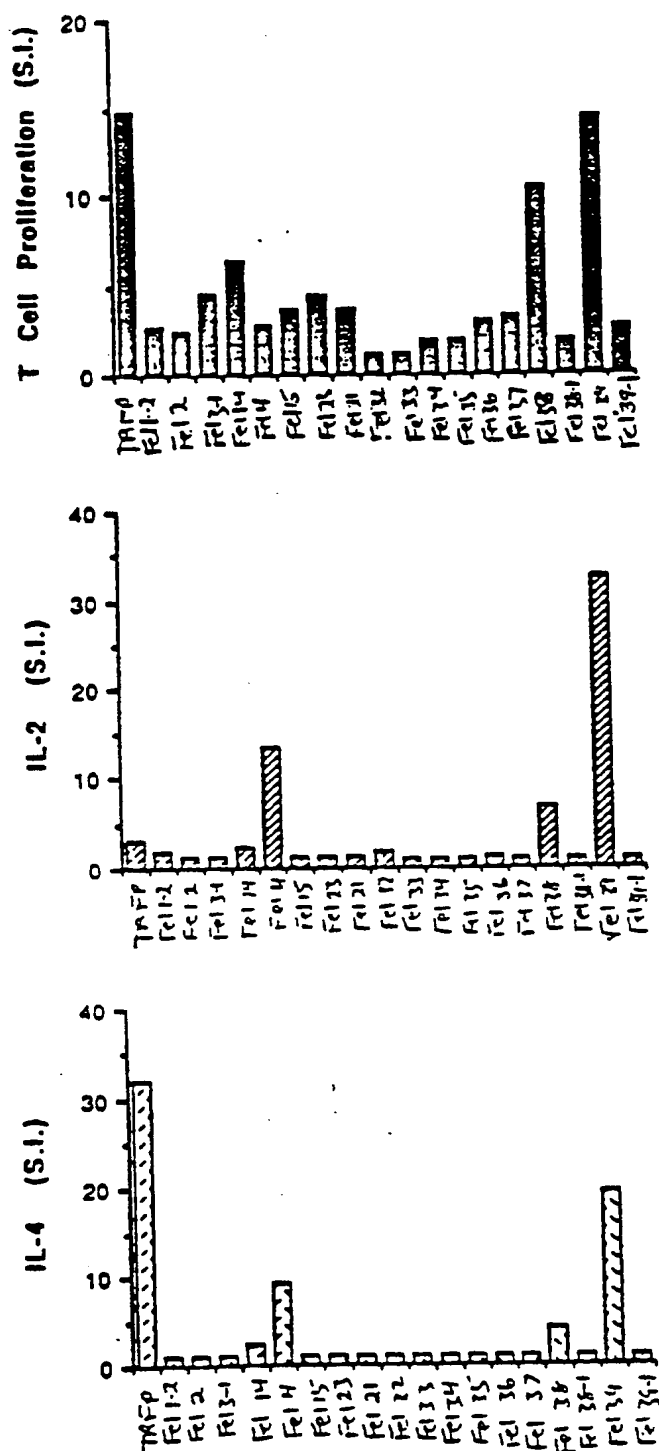


FIG. 17

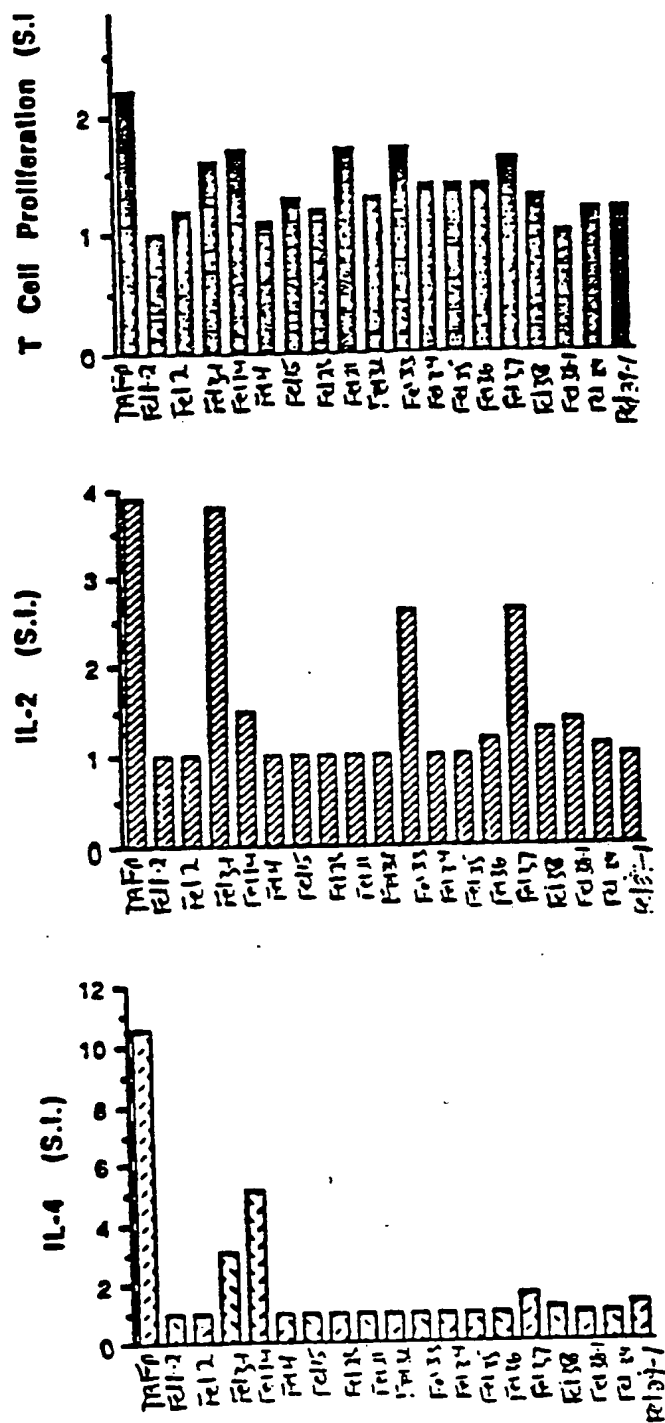


FIG. 18

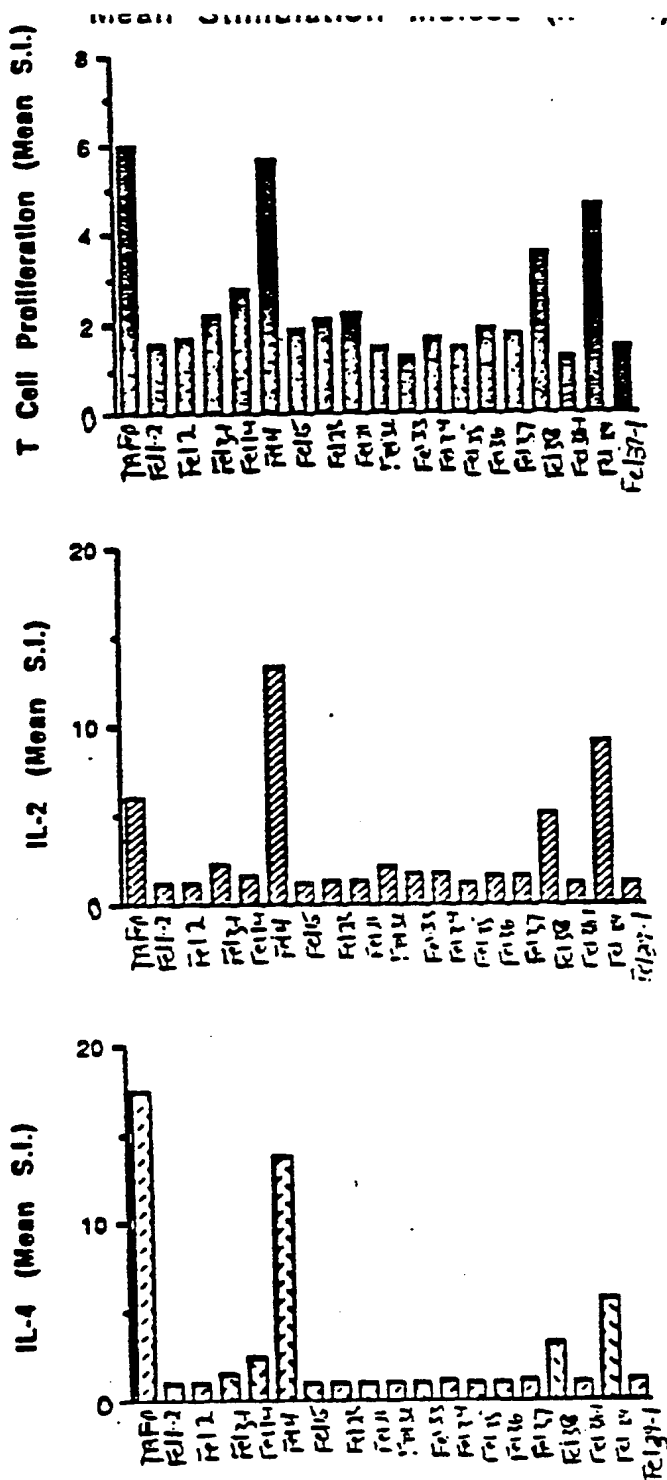


FIG. 19

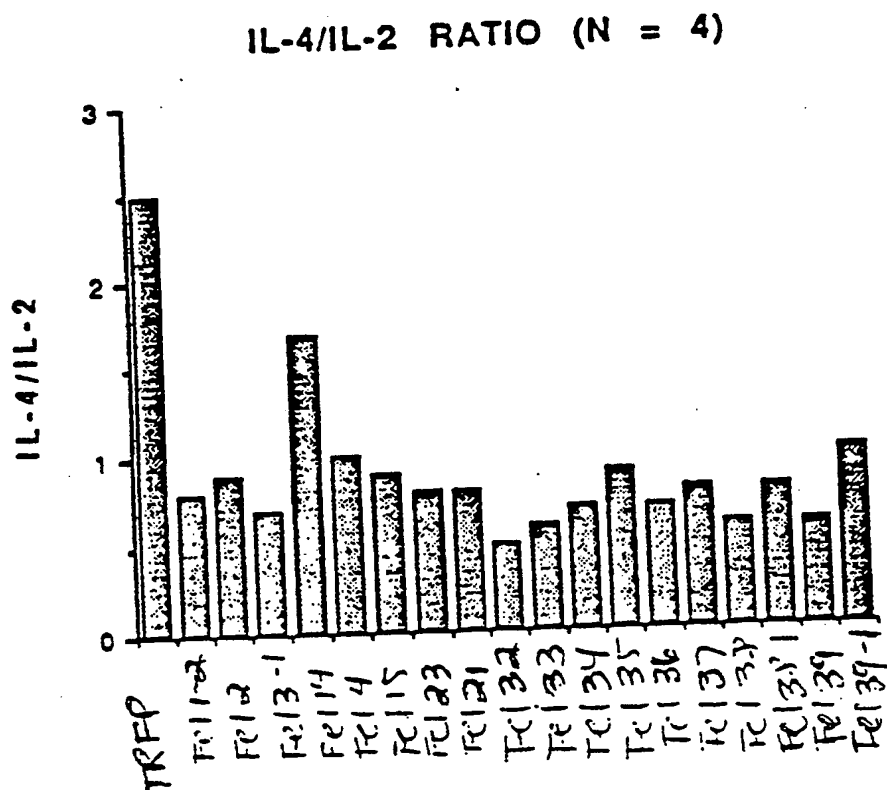


FIG. 20

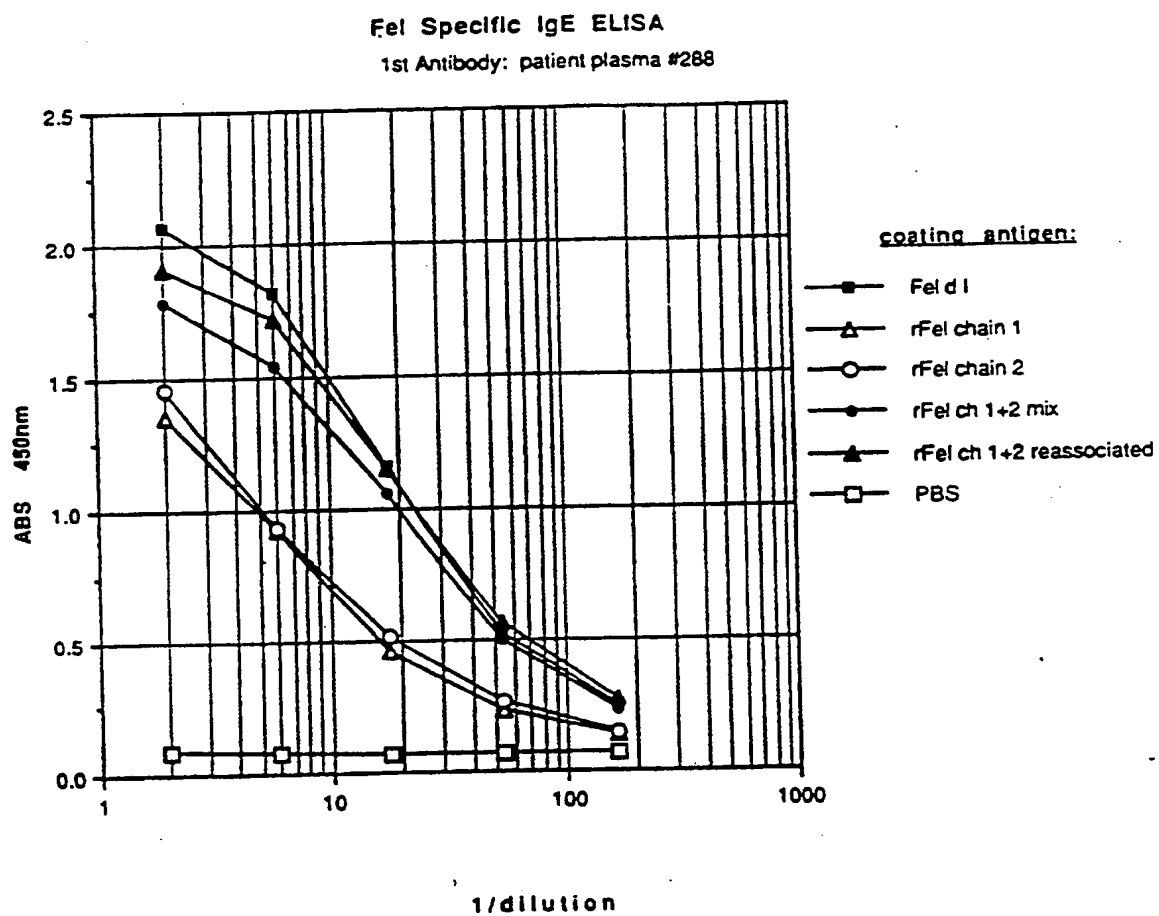


FIG. 21

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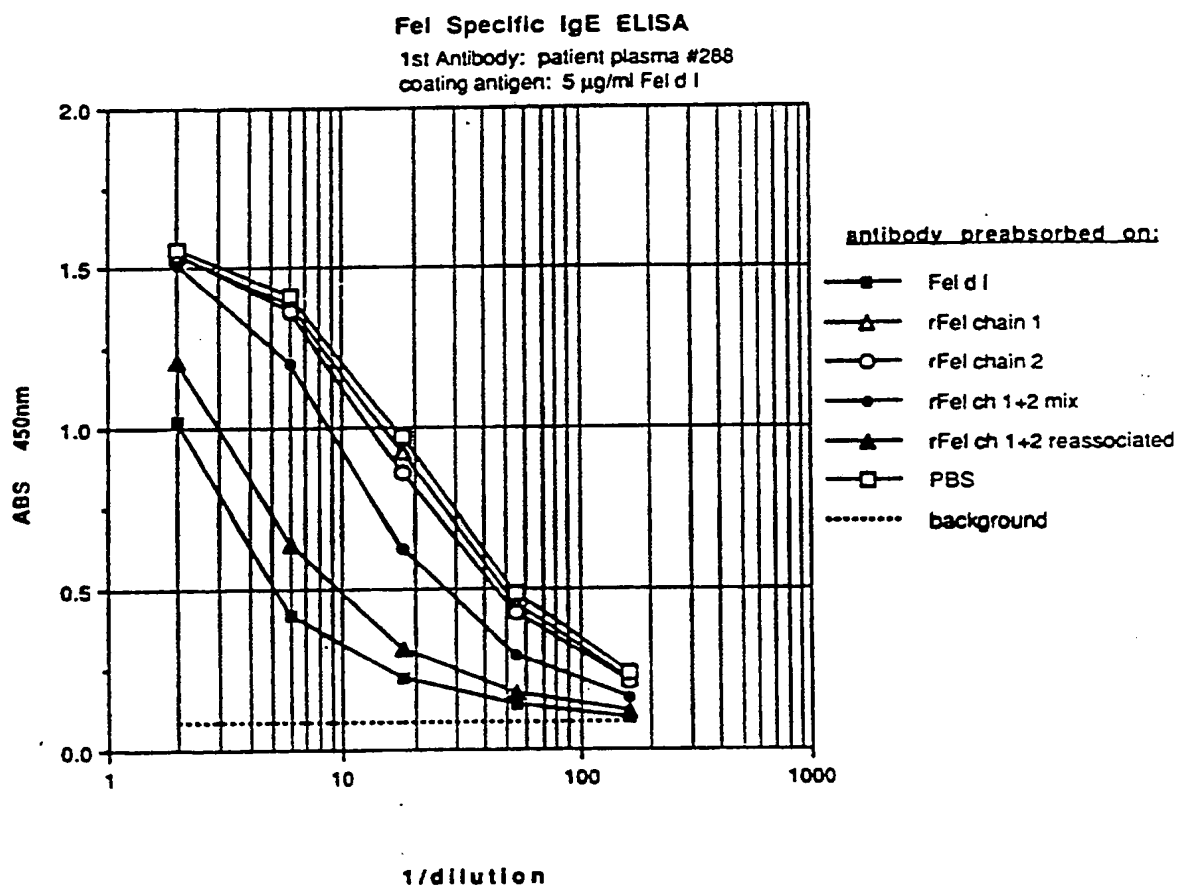


FIG. 2.2



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/12, A61K 39/35, C12N 15/62		A3	(11) International Publication Number: WO 93/19178
			(43) International Publication Date: 30 September 1993 (30.09.93)
(21) International Application Number: PCT/US93/02462		(74) Agents: MANDRAGOURAS, Amy, E.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US) et al.	
(22) International Filing Date: 25 March 1993 (25.03.93)		(81) Designated States: AU, CA, FI, HU, JP, KP, KR, NO, NZ, PT, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority data:		Published	
07/857,311	25 March 1992 (25.03.92) US	<i>With international search report.</i>	
07/884,718	15 May 1992 (15.05.92) US	<i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
08/006,116	15 January 1993 (15.01.93) US		
(71) Applicant: IMMUNOLOGIC PHARMACEUTICAL CORPORATION [US/US]; 610 Lincoln Street, Waltham, MA 02154 (US).		(88) Date of publication of the international search report: 25 November 1993 (25.11.93)	
(72) Inventors: GEFTER, Malcolm, L. ; 27 Coburn Road, Weston, MA 02193 (US). GARMAN, Richard, D. ; 86 Clarendon Avenue, Somerville, MA 02144 (US). GREENSTEIN, Julia, L. ; 174 Mount Vernon Street, West Newton, MA 02165 (US). KUO, Mei-chang ; 5 Cox Road, Winchester, MA 01890 (US). BRINER, Thomas, J. ; 438 Appleton Street, Arlington, MA 02174 (US). MORVILLE, Malcolm ; 17 Twin Lakes Drive, Waterford, CT 06385 (US).			

(54) Title: PEPTIDES USEFUL FOR INDUCING TOLERANCE

SEQUENCES

peptide
NAME

X	KRDVDLFLTGTPDEYVEQVAQYKALPV
Y	KALPVVLENARILKNCVDAMQTEEDKE
Z	YFAVANGHELLLDLSLTQVHATEPER
A	EEDKENALSLLDKIYTSPL
B	MGEAVQHTVEDLKLNTLGR
C	EEDKENALSLLDKIYT
D	MAISLLDKIYTSPL
E	TEEDKENALSLLDKIYTSPL

(57) Abstract

The present invention is based on the discovery that subcutaneous administration of a peptide comprising at least one T cell epitope of a protein allergen or other protein antigen results in T cell tolerance upon subsequent exposure to the allergen or other antigen. Peptides useful for inducing tolerance in a mammal, such as a human by subcutaneous administration or other routes of administration are within the scope of this invention. Such peptides include peptide X (SEQ ID NO: 7), peptide Y (SEQ ID NO: 8), peptide Z (SEQ ID NO: 9), peptide A (SEQ ID NO: 10), peptide B (SEQ ID NO: 11), peptide C (SEQ ID NO: 12), peptide D (SEQ ID NO: 13), and peptide E (SEQ ID NO: 14), each as shown in the figure. It is preferred that such peptides be administered in soluble form.

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INTERNATIONAL SEARCH REPORT

PCT/US 93/02462

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.C1. 5 C12N15/12; A61K39/35; C12N15/62		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.C1. 5	C12N ; C07K	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO,A,9 106 571 (IMMULOGIC PHARMACEUTICAL CORPORATION) 16 May 1991 see figure 6; example 6; table 4 ---	1-33, 37-44
E	WO,A,9 308 280 (IMMULOGIC PHARMACEUTICAL CORPORATION) 29 April 1993 see the whole document ---	1-33, 37-44
P,X	WO,A,9 215 613 (IMMULOGIC PHARMACEUTICAL CORPORATION) 17 September 1992 see the whole document -----	34-36
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IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
13 AUGUST 1993		23 -09- 1993
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		CUPIDO M.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/ 02462

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 13-24 and 29-44 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9302462
SA 71997

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CA-A- 2073045	04-05-91
		EP-A- 0500785	02-09-92
		JP-T- 5502445	28-04-93

WO-A-9308280	29-04-93	WO-A- 9308279	29-04-93

WO-A-9215613	17-09-92	AU-A- 1575192	06-10-92
